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THE INCREASE IN DEATHS ATTRIBUTED TO CANCER OF THE LUNG.

J. A. HEADY AND E. L. KENNAWAY.

From the Pathological Department, St. Bartholomew's Hospital, London, E.C.1.

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THE question, whether the recent increase in deaths attributed to cancer of the lung is due to a real increase in the incidence of this disease, has been discussed by many writers. The present paper, which brings forward no new data, is an examination of some of the evidence offered on this subject.

(1) *The Application of Statistics from Hospitals.*

The problem of finding the best index of a real increase in the incidence of lung cancer from hospital statistics is a difficult one.

The relevant facts usually ascertainable at a hospital are: The annual total of—

1. Admissions for all conditions.
2. Admissions for all cancer.
3. Admissions for cancer of the lung.
4. Deaths from all conditions.
5. Deaths from all cancer.
6. Deaths from cancer of the lung.
7. Post-mortems performed.
8. Post-mortems where cancer was found.
9. Post-mortems where cancer of the lung was found.

If an index is to be constructed showing a change in the incidence of cancer of the lung, it will consist of the ratio of two quantities—one showing, or purporting to show, the number of cases of cancer of the lung, and the other representing the population at risk.

The conditions which an ideal index should satisfy are:

(*a*) Both quantities must be reliable measures of what they are alleged to be.

(*b*) The number representing the population at risk must not vary from year to year in its quality—either of being representative of the general population, or of being likely to produce cases of cancer of the lung when judged by all the factors which are now suspected of influencing the incidence, such as age and sex.

(*c*) The number representing cases of cancer of the lung must bear a constant relationship to the true incidence in the population.

Of the factors enumerated above, Nos. 3, 6 and 9 could be used as a measure of the incidence of lung cancer. 3 and 6 are open to objection on the grounds of possibly mistaken diagnosis, hence presumably 9 is the most accurate measure of what it is stated to be.

As a measure of the population at risk, i.e. of becoming a case on which a post-mortem for cancer of the lung is done at a given hospital, we could use 1, 2, 4, 5, 7 or 8.

Do these measures change in their quality of being representative of the general population? The answer is that they all do so, because the cross-section of the population being admitted to hospital, whether of all cases or of cancer alone, changes, and therefore the cross-section of all deaths represented by deaths in hospital changes also. The assertion that the cross-section changes is based on knowledge that many people are now admitted to hospital who never would have been so admitted in the past—e.g. for investigation, or pregnancy. It is supported by the rapid decline in the ratio of deaths to admissions shown by the figures of Passey and Holmes (1935, Table II) for Liverpool Royal Infirmary and the London Hospital, which decline is unlikely to be due entirely to improved treatment.

Deaths as percentage of admissions.

	Liverpool.		London Hospital.	
	Male.	Female.	Male.	Female.
1904–1908 .	11·70	10·77 .	11·11	9·06
1924–1928 .	7·54	6·46 .	5·71	3·83

In other words, the average in-patient was, in 1928, less likely to die than he was about 1900. So that, if the incidence of cancer of the lung had remained constant in the general population, and the same policy of admission, and discharge before death, in cases of cancer of the lung, were adopted as before, the ratio of deaths from this form of cancer to total admissions would have fallen simply because of the change in type of admission. This argument rests on the assumption that a case of cancer of the lung that would be admitted in 1928 would have been admitted in 1900 and vice versa, i.e. that cancer of the lung is a sufficiently serious disease to warrant admission, however short the supply of beds. The same argument, that the cases would have been sufficiently serious to be admitted under any circumstances, could be applied, though with less certainty, to 2, 4, 5, 7, 8. Of these it is suggested that 7 is the most satisfactory population "at risk" from this point of view, because it is less dependent than is 8 on any increase in the incidence of cancer as a whole, and will automatically compensate for an ageing population.

How accurately the number of hospital post-mortems where cancer of the lung was found would reflect an actual increase in cases of cancer of the lung in the general population must depend on at least two factors as well as those enumerated above—namely: (*a*) whether the number of hospital deaths from cancer of the lung is a constant proportion of the number of admissions for that condition, and (*b*) whether the proportion of deaths from cancer of the lung coming to post-mortem is constant over the years.

By choosing the total number of post-mortems as a population "at risk" we hope partially to eliminate the effect of factor (*b*) because the corresponding

factor should equally affect the population at risk, though we cannot, of course, correct for any deliberate selection of cases of cancer of the lung for autopsy on account of their intrinsic interest.

We can find no evidence on factor (*a*) taken separately, while the evidence on factor (*b*) is indirect. The proportion of all hospital deaths coming to post-mortem certainly shows no increase if we accept Passey and Holmes' (1935) figures as shown in their Table I and reproduced in part below as our Table I.

TABLE I.—*Total Post-mortems as Percentage of Total Deaths in Certain Hospitals (Passey and Holmes).*

Five-year period.	Total deaths.	Total post-mortems.	Post-mortems as per cent of deaths.
1894–98	25,815	16,534	64·4
1899–1903	30,744	18,822	61·2
1904–08	34,073	21,821	64·0
1909–13	46,234	28,637	61·0
1914–18	43,926	24,098	54·8
1919–23	43,971	23,726	53·9
1924–28	44,998	26,110	58·0

Kennaway and Kennaway (1947) record that according to the death certificates, the percentage of deaths from cancer of the lung in England and Wales where a post-mortem was performed was 25·0 for the period 1921–32, and 24·6 for the period 1933–38, which data give no indication of any change.

By using figures from Bonser's (1934) Tables I and V we arrive at Table II:

TABLE II.—*Post-mortems for Cancer of the Lung as Percentage of Cases of Cancer of the Lung Diagnosed in the Wards of Leeds General Infirmary (Bonser, 1934).*

Five-year period.	Cases of cancer of the lung diagnosed in the wards. (A)	Cases of cancer of the lung at post-mortem. (B)	Percentage (B) of (A)
1918–22	61	24	39
1923–27	137	44	32
1928–32	160	50	31

This table gives evidence on both factors (*a*) and (*b*) above taken together, and admittedly is very scanty. Unfortunately it seems to be the only such evidence available. There is certainly no sign of any dramatic change.

Table III (calculated from the data given by Passey and Holmes (1935)) gives the percentage which intrathoracic cancer at autopsy formed of all autopsies in certain representative voluntary hospitals in Great Britain. This is the ratio suggested above as probably the best obtainable from hospital statistics as an index of the rise or fall of lung cancer in the general population. Any tendency to rise occurs in the last two five-year periods, i.e. from 1919 onwards, which is in general accord with the rise shown in Fig. 1.

TABLE III.—*Intrathoracic Cancer at Autopsy as Percentage of all Autopsies at Certain British Hospitals (Passey and Holmes).*

Five-year period.	All autopsies (A).	Intrathoracic cancer at autopsy (B).	Percentage (B) of (A).
1894–98	16,534	173	1·05
1899–1903	18,822	180	0·96
1904–08	21,821	234	1·07
1909–13	28,637	331	1·16
1914–18	24,098	257	1·07
1919–23	23,726	386	1·63
1924–28	26,110	552	2·11

It is interesting to compare the figures for post-mortems where cancer of the lung was found in the hospitals investigated by Passey and Holmes, with the Registrar-General's figures for deaths from cancer of the lung in England and Wales in the same period (Table IV).

TABLE IV.—*Ratio of Autopsies where Cancer of the Lung was found in Certain Hospitals to Deaths attributed to Cancer of the Lung in England and Wales (Passey and Holmes, and Registrar-General).*

Five-year period.	Hospital autopsies with cancer of the lung (Passey and Holmes) (A).	Registrar-General's figures for deaths from cancer of lung (B).	Mean annual increase.	Ratio (A)/(B).
1899–1903	180	1234	..	0·146
1904–08	234	1524	58	0·154
1909–13	331	2062	108	0·161
1914–18	257	2072	2	0·124
1919–23	386	2719	129	0·142
1924–28	552	4444	345	0·124

This ratio shows no systematic change, and can be regarded as very stable considering the uncertainties and small numbers involved. The two measures, hospital autopsies with cancer of the lung and the Registrar-General's figures for deaths attributed to cancer of the lung, would therefore appear to vary together, and one can place as much, or as little, confidence in the one as in the other.

Possible conclusions are :

(*a*) The whole question of assessing a change in the incidence of cancer of the lung in the general population from hospital statistics is difficult, and any conclusions must be treated with the utmost reserve.

(*b*) Of the possible indices from hospital figures the ratio of autopsies where cancer of the lung was found to total autopsies is likely to be the best indication of any such change.

(*c*) Any ratio which uses the total number of admissions as a population at risk is likely to be misleading because the character of the hospital population is changing.

(2) *Evidence from British Hospitals.*

Willis (1948) says: "Of significance are the analyses of necropsy records made by Bonser (1928 and 1934) and by Passey and Holmes (1935). Bonser's analysis of the necropsies during 41 years at Leeds, where an unusually high proportion of fatal cases were examined, showed no increase in the incidence of

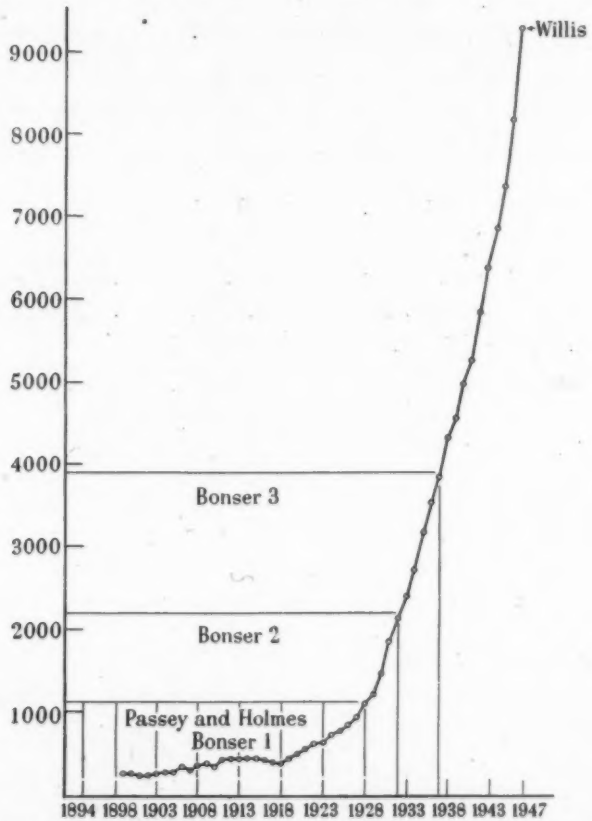

FIG. 1.—Cancer of lung. Death certificates. England and Wales, 1899–1947.

intrathoracic cancer when considered with respect either to the total number of necropsies, the total number of cancer cases, or the total number of admissions to hospital. Passey and Holmes studied the incidence of intrathoracic cancer in the necropsy records of 16 major teaching hospitals in Great Britain: in 8 hospitals there was no evidence that this was increasing, in 3 the results were inconclusive, while in 5 institutions which did show an increase there were special circumstances which may have been responsible. Sitsen (1935, *Zeit. Krebsforsch.*, **42**, 30) and Steiner (1944, *Arch. Path.*, **37**, 185) also are among the many

pathologists who deny that there is any satisfactory evidence of a real increase in the incidence of lung cancer during recent years. The suspicion is that where such increase has appeared to have been conspicuous, there was formerly a low standard of accuracy of pathological diagnosis, and that the standard has improved with the passage of time."

"For the foregoing reasons, comparisons of early and recent clinical or necropsy estimates of incidence, or comparisons of the findings in different countries or in different hospitals, must be quite unreliable. So much depends on the personal experience of the clinicians and pathologists concerned, and current journals contain evidence enough that a uniformly high standard of diagnosis of this elusive disease has not yet been attained by either. Now that the properties of the disease are becoming better known, however, its true frequency and trend in a given community or institution might be ascertainable by meticulously careful and complete necropsies performed by skilled pathologists on all fatal cases over a period (probably 20 or 30 years) sufficient to obviate chance fluctuation."

TABLE V.—*Cancer of Lung. Death Certificates. England and Wales.* 1899–1947.

Year.	Men.	Women.	Total.	Year.	Men.	Women.	Total.	Year.	Men.	Women.	Total.
1899	139	92	231	1915	258	168	426	1932	1553	565	2118
1900	152	102	254	1916	269	144	413	1933	1820	582	2402
1901	120	97	217	1917	250	152	402	1934	2095	680	2775
1902	128	121	249	1918	257	141	398	1935	2337	858	3195
1903	157	126	283	1919	255	175	430	1936	2606	917	3523
1904	165	125	290	1920	309	191	500	1937	2914	927	3841
1905	146	124	270	1921	361	186	547	1938	3273	1049	4322
1906	192	130	322	1922	423	189	612	1939	3391	1149	4540
1907	164	133	297	1923	405	225	630	1940	3808	1180	4988
1908	203	142	345	1924	493	233	726	1941	4069	1156	5225
1909	207	176	383	1925	508	276	784	1942	4579	1267	5846
1910	206	143	349	1926	578	272	850	1943	5081	1296	6377
1911	261	175	436	1927	666	290	956	1944	5491	1360	6851
1912	265	188	453	1928	814	314	1128	1945	5982	1480	7462
1913	263	178	441	1929	849	359	1208	1946	6765	1430	8195
1914	252	181	433	1930	1056	433	1489	1947	7667	1620	9287
				1931	1358	522	1880				

Does the very destructive estimate of hospital statistics as "quite unreliable" in the second paragraph quoted above apply to the data in the preceding paragraph when these show no increase in cancer of the lung?

One might suggest that Willis is placing upon the material of Passey and Holmes a significance which it is not fitted to bear. For many years some students of this subject have wondered whether the (alleged) absence of any evidence in their data of an increase in cancer of the lung was not due to the fact that the great bulk of this increase took place after the period studied by Passey and Holmes (Fig. 1 and Table V). Willis does not point out that between the last year of that period (1928), and the year in which, presumably, he wrote (1947), the deaths attributed annually to cancer of the lung in men in England and Wales rose no less than ninefold (from 814 to 7,667), and those of women from 314 to 1,620; one might expect some comment upon this enormous increase.

Passey and Holmes (1935) summarize their results in their Table VI bearing the title, "Combined Figures of the Hospitals in Tables III, IV and V for the years 1894 to 1928 inclusive, showing Percentage Incidence in Admissions, in Deaths, and in Autopsies." This table is reproduced *in extenso* here (Table VI).

TABLE VI.—*Intrathoracic Cancers at Autopsy as Percentage of Hospital Admissions (Passey and Holmes).*

Five-year period.	Total admissions.	Total intrathoracic cancers at autopsy.	Percentage in admissions.
1894–98	315,059	173	0·055
1899–1903	374,207	180	0·048
1904–08	420,595	234	0·056
1909–13	597,193	331	0·055
1914–18	625,833	257	0·041
1919–23	671,846	386	0·057
1924–28	786,912	552	0·070

Passey and Holmes comment upon this table as follows (italics not in the original):

"... the incidence percentage which was 0·055 in the beginning of the thirty-five-year period was still but 0·057 in the 1919–23 period, showing a rise of less than 4 per cent. *Practically the whole of the rise which has taken place in the thirty-five years has occurred during the last five years of the inquiry.*"

"It is extremely unlikely that the increase shown in the last five-year period indicates a real increase. In the first place, practically the whole of it is accounted for by five only out of the sixteen centres. Moreover, in the case of each of these five centres special features have been indicated which might well have had an influence on their statistics; at least it would not be wise to exclude the possibility. *Again, such rise as is shown is not gradual, but occurs as a sudden jump in the last five-year period.* While reasons have been advanced which offer some explanation for the sudden rise in these five centres, it is probable too that increased interest in the condition and an improved standard of diagnosis have at the same time augmented these influences."

Reasons have been set down above why the ratio of intrathoracic cancer at autopsy to total admissions cannot be accepted as a good indication of a real change in incidence of cancer of the lung. Accepting, however, for the moment the figures in Table VI at their face value, the sudden jump in 1924–28 is what is shown at about that time by the figures for deaths attributed to cancer of the lung in England and Wales, the mean annual increase (Table IV) rising from 129 deaths (1919–23) to 345 (1924–28). It is also in good agreement with the figures shown in Table III.

Willis, in the passage quoted above, says that "in 5 institutions which did show an increase there were special circumstances which may have been responsible." These five hospitals are, in London, St. Bartholomew's, St. Mary's, University College and the London Hospital, and the General and Queen's Hospitals in Birmingham (Passey and Holmes, 1935). A tuberculosis dispensary had been established at the first three of these hospitals; a similar change at St. Thomas's Hospital was not accompanied by any increase, and for this exception reasons are found. At the London Hospital (Passey and Holmes, 1935) "certain physicians were undoubtedly interested after the War in thoracic neoplasms"; this fact would be of greater value as evidence if we could be told that there were no such physicians at the other ten hospitals (Aberdeen Royal Infirmary, Edinburgh Royal Infirmary, Glasgow Royal Infirmary, Glasgow Western

Infirmary, Guy's Hospital, Leeds General Infirmary, Liverpool Royal Infirmary, Manchester Royal Infirmary, St. George's Hospital, Sheffield Royal Infirmary).

Thus the data recorded in the very careful and elaborate study of Passey and Holmes, when put forward 20 years later as representing the state of our knowledge to-day, appear to show the unavoidable defect that the investigation ended in 1928, when the rapid increase of the last 20 years was just beginning.

Bonser has published three papers (1929, 1934, 1938) on this subject, referred to in Fig. 1 as 1, 2, 3, dealing with the cases of intrathoracic cancer coming to autopsy at Leeds General Infirmary during three periods, namely :

Period 1—1891–1927 (32 years' records available) .		172 cases.
,, 2—1928–32		50 ,,
,, 3—1933–37		72 ,,
		294 cases

No attempt is made here to present the large amount of material collected by Bonser from other hospitals.

Bonser draws the following conclusions :

Period 1.—" There has been no increase of intrathoracic cancer at post-mortem in Leeds during the last 35 years " (1929).

Period 2.—" . . . there has been no increase during 41 years in the incidence of intrathoracic cancer compared with total post-mortems, total cancers or total admissions to hospital " (1934), though " a very slight increase in 1928–32, which is unlikely to be of significance unless sustained for several more years," is noted.

Period 3.—The results for the whole period 1891–1937 are summarized in a table of which a part is reproduced verbatim in Table VII : "After a long period of relatively steady incidence of intrathoracic cancer at post-mortem at the Leeds General Infirmary from 1891–1927, there has been a considerable increase in the last 10 years. This increase is more noticeable in the last five years, and is seen whether intrathoracic cancers are reckoned in relation to total post-mortems, total cancers or total admissions to the hospital."

" No evidence is obtainable to show whether the sample of the population admitted to the Leeds Infirmary has undergone a very serious alteration in the last ten years. Greater clinical interest in intrathoracic cancer and the hope of surgical cure may have increased the number of admissions of this type of case. There is evidence to show that the sample of cases which come to post-mortem has not undergone any special alteration, and as a high percentage of deaths is examined selection of cases on account of their special interest is not frequent."

Willis does not refer to this third paper, either in the text or in the bibliography.

The figures in Table V show that the annual deaths attributed to cancer of the lung in Bonser's second period rose from 1128 (1928) to 2118 (1932), or by 990, and in the third period from 2402 (1933) to 3841 (1937), or by 1439.

TABLE VII.—*Intrathoracic Cancer at Post-mortem. Leeds General Infirmary (Bonser, 1938).*

Year.	Total.	Male.	Female.	Per cent of all post-mortems.	Per cent of all cancers.	Per cent of all admissions.	Total admissions to wards of hospital.
1891–92*	2	2	0	0·81	6·30	0·039	5,125
1893–97*	10†	6	3	1·05	7·42	0·058	17,276
1898–1902*	12	9	3	1·12	6·60	0·065	18,443
1903–07	18	15	3	0·86	4·95	0·055	32,740
1908–12	31	23	8	1·27	8·27	0·085	36,368
1913–17	31	22	9	1·30	7·73	0·066	46,654
1918–22	24	18	6	0·91	6·17	0·052	46,410
1923–27	44	34	10	1·17	7·97	0·074	59,845
1928–32	50	39	11	1·38	7·50	0·073	68,586
1933–37	72	64	8	2·31	11·32	0·106	68,089

* The records of some of these years were missing.
† The sex in one of these cases was not stated.

(3) *Evidence from the Central Tuberculosis Station, Copenhagen (Clemmesen and Busk).*

Clemmesen and Busk (1947), in a paper entitled "On the Apparent Increase in the Incidence of Lung Cancer in Denmark, 1931–45," give the actual numbers of deaths for males and females, and the crude mortality rates, in Copenhagen, provincial towns, rural areas, and the whole country. The totals are shown below in Table VIII.

TABLE VIII.—*Deaths from Pulmonary Cancer in Denmark (Clemmesen and Busk).*

	1931–35.	1936–40.	1941–45.
Males	264	494	906
Females	180	215	369

The authors go on to present a graph of peculiar form (their Fig. 3), which appears to be the chief result of the investigation. The data from which the graph is constructed consist of the cases of lung cancer detected at the Central Tuberculosis Station, Copenhagen, among patients, suffering from respiratory diseases, referred by general practitioners; they are contained in their Table II (reproduced here in part as Table IX).

TABLE IX.—*Pulmonary Cancer. Central Tuberculosis Station, Copenhagen (Clemmesen and Busk).*

	Males over 45.			Females over 45.		
Year.	Examined.	Cancer.	Morbidity rate per 10,000.	Examined.	Cancer.	Morbidity rate per 10,000.
1936	466	7	150·2	487	0	—
1937	523	5	95·7	578	2	36·1†
1938	526	5	95·2	581	1	17·2
1939	630	7	111·0	667	1	15·0
1940	568	6	105·6	627	1	16·0
1941	696	20	291·9*	690	1	14·5
1942	916	13	141·8	878	2	22·8
1943	967	20	206·8	1055	4	37·9
1944	1056	13	123·3	1016	2	19·7
1945	1057	14	132·5	1129	1	8·9

* This figure should be 287·3.
† This figure should be 34·6.

The authors say that ". . . the curve for males examined given in Fig. 3 shows no steady rise . . . ," which is perfectly true, and they conclude: " In our opinion the figures given illustrate that even a pronounced increase of the crude mortality rate for lung cancer among males, and almost only among males, does not necessarily mean an increase in incidence of that disease. Presumably the more frequent detection of the disease is due to improvement of diagnostic procedures, and increased attention, and similar explanations may be correct in the case of occupations with an increased mortality from lung cancer."

One has difficulty in believing that any generalizations about cancer of the lung can be based upon such a peculiar succession of such small totals, drawn from a highly selected population, as those given in the third column of the table, namely 7, 5, 5, 7, 6, 20, 13, 20, 13, 14 cases of pulmonary cancer. Is one to believe that the totals 6, 20, 13 in the three consecutive years 1940, 1941 and 1942 correspond to any similar annual incidence upon the whole population of Denmark ? If there is no such parallelism, what is the value of these figures, and how can they provide a basis for the important generalization quoted above ?

One may doubt whether the radiographic examination of any population for pulmonary tuberculosis adds much to our knowledge of the total incidence of cancer of the lung. Such a survey will, of course, reveal a number of cases, previously undetected, of such cancer. But it does not follow that any large number of these cases would have remained undetected, and unrecorded on the death certificates, by the time that these persons had gone through the usual course of dying of this form of cancer.

SUMMARY.

The problem of assessing from hospital statistics any change in the incidence of cancer of the lung upon the whole population is discussed. The ratio of autopsies for cancer of the lung to total autopsies is suggested as the best index of such change. Some data from British and Danish sources are examined.

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REFERENCES.

BONSER, G. M.—(1929) *J. Hyg.*, **28**, 340.—(1934) *Ibid.*, **34**, 218.—(1938) *Acta int. Un. Cancer*, **3**, 119.

CLEMMESEN, J., AND BUSK, T.—(1947) *Brit. J. Cancer*, **1**, 253.

KENNAWAY, E. L., AND KENNAWAY, N. M.—(1947) *Ibid.*, **1**, 260.

PASSEY, R. D., AND HOLMES, J. McD.—(1935) *Quart. J. Med.*, N.S., **4**, 321.

Registrar-General's Statistical Review of England and Wales. Text. London: H.M. Stationery Office.

WILLIS, R. A.—(1948) ' Pathology of Tumours.' London : Butterworth.

FAMILIAL CARCINOMA OF THE DUODENUM IN ADOLESCENCE.

H. UNGAR.

From the Department of Pathology, Hadassah University Hospital, Jerusalem, Israel.

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CARCINOMA of the duodenum has been observed in this laboratory at autopsy in three young siblings whose case reports form the subject of this paper. The relation of these cases to generalized intestinal polyposis will be discussed.

CASE I.

Rivkah A—, aged 19, was admitted to the Hadassah University Hospital in January, 1939.

Previous history.—The patient suffered from malaria and typhoid fever several years ago. In 1935 she experienced a sudden pain in her right eye; exophthalmos later developed on the same side. At examination in the Eye Clinic of the Hadassah Medical Organization "endothelioma" of the orbita was diagnosed. No biopsy was made.

Recent history.—Two months prior to admission to the hospital, patient complained of attacks of pain in the upper part of the abdomen. A blood count revealed considerable secondary anaemia. During 5 weeks prior to admission progressive jaundice was present.

Two days before admission the temperature rose to 40° C., and a chill was experienced.

During the fortnight between admission and death there was intermittent fever, the temperature ranging between 38° and 40° C. During the same period, the jaundice subsided notably. Abdominal pain attacks occurred with increasing frequency and intensity. There were repeated attacks of chills; blood culture revealed *Staphylococcus aureus*. Terminally, bronchopneumonia developed. The final clinical diagnosis was as follows: Septicaemia. Obstructive jaundice, possibly due to pressure of enlarged lymph nodes. Systemic disease of the lymphatic system, ? Hodgkin's disease.

Autopsy Findings.

Anatomical diagnosis.

Adenocarcinoma and papilliferous carcinoma of the duodenum (surrounding the papilla of Vater); hepatic abscesses due to ascending infection of bile ducts. Papillary malignant adenoma of duodenum. Multiple polyps of colon. Extrabulbar tumour of right orbita (clinical diagnosis "endothelioma"). Cavernous haemangioma of the base of tongue.

Record of anatomical findings (abbreviated).

Autopsy was performed 3 hours after death by evisceration through a small incision below the right costal arch. The pertinent findings were as follows:

Duodenum (Fig. 1).—In the area of the papilla of Vater, there was a wide defect with a depressed centre whose floor was torn and rugged. The defect

measured 3-4 cm. in diameter. The elevated edges were in part undermined, showing papilliform lobulations. The entire area was adherent to the pancreas. In sections through the centre of the ulceration the layers of the duodenal wall were found obliterated but scarcely thickened. Between the duodenum and pancreas a layer of firm whitish tissue was present.

Separated from the tumour by a small segment of normal mucosa, there was a pedunculated node about the size of a walnut, the surface of which exhibited papillary formations. Within the same segment of the intestine, a second pedunculated pea-sized node of similar appearance was found.

In the second and third portion of the duodenum numerous small formations which appeared in some areas as small polyps and in other areas as tiny ridges were found (Fig. 4). These formations measured between 2 and 4 mm. in diameter. They were localized either between the plicae or at their height. They were found either singly or crowded together.

Jejuno-ileum.—Without pathological changes.

Colon.—The mucosa of the transverse colon contained two pea-sized polyps which were rather firm and could be shifted with ease on the underlying tissues.

Liver.—Weight 2090 g. Marked enlargement. Cross-sections revealed in the upper part of the right lobe several cavities in irregular distribution which measured 1 to 4 cm. in diameter and contained a thick yellowish pus. The walls of the cavities were lined by a membrane-like yellowish layer, about 1 mm. in width. The surrounding liver tissue was a yellowish-brown colour. The acini were distinctly marked.

Gall bladder.—Without changes. Bile ducts throughout their whole extent without changes.

Histological Examination.

1. *Sections from the ulcerated tumour.*—Within wide necrotic areas there were islets of columnar-cell carcinoma which in some areas assumed the character of adenocarcinoma. The occasionally papillary architecture was suggestive of a tumour which could have originated in a papillary adenoma (Fig. 5). In other areas the carcinoma infiltrated into the muscular layers in solid strands but did not invade the pancreas. Epithelium presented marked pleomorphism. The stroma was sparsely developed ; it was infiltrated by scattered small round cells.

2. *Small polypoid formations in the duodenum* were examined in several blocks from different areas. The examination revealed several histological aspects which may be regarded as representative of successive stages in the development of polyps.

In the area of the prominence, the submucosa was widened and the muscularis mucosae showed a funnel-shaped protrusion in the direction of the intestinal lumen. The whole formation was covered by a mucous membrane which was not different from the surrounding mucosa. Other polyps were chiefly formed by hypertrophic mucosa and only near the base was the submucosa slightly thickened.

3. *Fragments from the two largest papillary polyps in the duodenum* (Fig. 6).—These polyps were built upon an axis of loose connective tissue, fat tissue and a muscularis mucosae which ramified into papillae. The glands were crowded together ; their ramified lumina showed variations in width and tortuosity ; frequently papillary projections into the lumen were seen. Groups of condensed

glandular formations often assumed a nodular form and in some instances were outlined by a thin layer of smooth musculature. In some places the glandular epithelia were piled up ; they did not reach beyond the base of the glands. The nuclei were dark-staining and columnar. The cytoplasm was scanty and dark-oxyphilic. Scattered mitotic figures were present. Toward the base of the papilloma, the epithelium assumed a more quiet character and gradually goblet cells became more numerous. In the continuation, there were Brunner's glands without abnormalities.

The irregular hyperplasia of the glands, the lack of goblet cells, the dark-staining nuclei and the appearance of typical and atypical mitotic figures justify the diagnosis of an early malignant change.

4. *Polyp of colon* (Fig. 7) was entirely composed of mucosal glands which were irregularly wide. Their epithelium, like that of adjacent mucosa, was without abnormalities. The surface of the polyp was ulcerated and there was superficial granulation tissue.

Case II.

Chajim A—, aged 16 at the time of death. The earlier history contained no contributory information. Fourteen months prior to admission he first began to complain of bouts of cramp in the abdomen. These occurred at irregular intervals and were without clear localization.

The patient was admitted to the hospital in June, 1939, three months before his death. At admission his chief complaints were shortness of breath, attacks of tachycardia, lack of appetite and abdominal colic localized under the costal arch on either side. During the preceding year the patient had lost a great deal of weight. There were recurrent periods of diarrhoea. Marked secondary anaemia was found. Examination of stools showed ascaris and taenia ; occult blood was repeatedly present. Frank blood was seen in stools only a short time before death. X-ray examination of stomach and duodenum failed to reveal pathological changes.

The attacks of abdominal pain became more frequent and more intense during the patient's stay in the hospital. The patient died about three months after admission.

Autopsy Findings.

The autopsy was done 18 hours post mortem by a short abdominal incision. Signs of decomposition were then already in evidence.

Anatomical diagnosis.

Columnar-cell carcinoma of duodenum with partial change to carcinoma simplex, necrosis, ulceration and continuous invasion of regional and adjacent mesenteric lymph glands. Papillary adenoma of Lieberkuhns glands of duodenum. Multiple polyps of duodenum.

Record of anatomical findings (abbreviated).

Abdominal cavity.—Liver extended by 4 cm. below the right costal margin. Peritoneum was smooth and glistening. Small intestines were distended by

splashing contents. Caecum and colon showed considerable meteorism. Intestinal wall exhibited dark-red and greenish discolouration due to post-mortem changes.

Duodenum (Fig. 2).—At a distance of about 1·5 cm. behind the pylorus there was a walnut-sized polyp on a short and narrow pedicle. The duodenal mucosa as far as 1 cm. behind the papilla of Vater was without lesions. Choledochus and pancreatic duct without abnormalities.

Beyond the papilla of Vater, the lumen was considerably distended. There was a nearly circular, crateriform defect, which extended from the posterior wall, including almost the entire circumference of the duodenum. The lesion, which measured 7 to 8 cm. in diameter, showed a rugged floor; it was entirely composed of tumour tissue and blended underneath with enlarged lymphatic glands of the peripancreatic group. The latter were glued together and had been completely replaced by tumour. Sections through the floor of the ulceration revealed a firm and, in some places, a friable tissue which was of a pale greyish-yellowish colour. The tumour was defined against the head of the pancreas by a thin layer of greyish-red connective tissue.

The remaining portion of the duodenum was considerably distended and filled with greenish fluid. The mucosa was extremely pale; it exhibited several soft tiny polypoid or ridge-like elevations which could be moved with the mucosa. They were of a uniform size and only rarely as high as the plicae transversae.

Because of the advanced state of the post mortem changes, close examination of the remainder of the intestines was not attempted.

Abdominal lymph nodes.—The lumps of peripancreatic nodes which have been mentioned were completely fused with lymph nodes in the upper portion of the mesenteric root and around the adjacent portion of the inferior vena cava, both of which had been overrun by tumour tissue. Other mesenteric glands were free of pathological lesions.

Histological Examination.

1. *Sections from the large ulcerated tumour* revealed a carcinoma which grew in some areas in solid strands, while in others gland-like formations predominated. Epithelia showed a considerable degree of pleomorphism and there were numerous typical and atypical mitotic figures. Near the margin of the ulcerated portion, the tumour was seen to extend into muscular and submucosal layers.

2. *Pedunculated tumour between pylorus and papilla of Vater.*—This presented formations of a papilloma which was built around ramifications of submucosa and muscularis mucosae. The main mass of the node was made up of glandular tissue with regular lumina which in places were slightly tortuous. Epithelium was columnar with dark nuclei and oxyphilic cytoplasm. There was no pleomorphism or mitotic activity. No goblet cells were present.

Between the glands there was a fibrous stroma which in most areas was sparsely developed. It contained infiltrations of small round cells, plasma cells and scattered foci of polymorphonuclear leucocytes. Brunner's glands were without pathological lesions. In some places single dark-celled glands projected from the head of the papilloma between the acini of Brunner's glands.

3. *Duodenal wall with small polyps.*—The histological aspects of the small polyps closely resembled those observed in Case I.

Case III.

Joseph A—, aged 18, was admitted for the first time to the Hadassah University Hospital in August, 1944, with the diagnosis of acute appendicitis followed by perforation.

Previous history not contributory.

On operation, a fluctuating mass, which was believed to be a perityphlitic abscess, was found above the ileocaecal region. Appendix was not removed.

The wound was drained and healed within a month by secondary intention. Following laparotomy the patient lost rapidly in strength, and concomitantly a gradual increase in the size of the abdomen was noted. Temperature remained normal; bowel movements were regular.

Three months after the operation, the patient was re-admitted to the Hospital because of severe intestinal haemorrhage. A firm huge tumour mass was palpable in the right half of the abdomen extending from the costal arch to the height of the crista iliaca. Having regard to the familial history a probable diagnosis of duodenal carcinoma was made. Patient received repeated blood transfusions. He died nine days following admission.

Autopsy Findings.

Anatomical diagnosis.

Adenocarcinoma of the terminal portion of duodenum.

Metastatic carcinoma in parapancreatic and paraportal lymph nodes.

Chronic encapsulated peritoneal abscess resulting from two fistulae, on the floor of two ulcers (non-neoplastic) in the upper jejunum.

Acute generalized suppurative peritonitis due to perforation of the encapsulated peritoneal abscess.

Polyposis of small intestines; single polyps of colon.

Extreme anaemia of tissues; septic hyperplasia of spleen.

Terminal endocarditis of tricuspid and mitral valve.

Summary of autopsy record (1½ hours after death).

Body extremely emaciated. Pallor of skin and visible mucous membranes. Abdomen markedly distended and tense.

Abdominal cavity.—Entire peritoneum was covered by fibrinous deposits There were easily severable adhesions between intestines and the anterior abdominal wall, mostly on the right side. Only in the right upper quadrant were the adhesions firm and included a cavity, about the size of the grape-fruit, which was defined by the anterior wall of the duodenum, the transverse colon near the hepatic flexure, a small portion of the descending colon and several loops of the jejunum. The cavity, which contained a dark feculant fluid, connected with the rest of the peritoneal sac by a small rent in the direction of the caecum. Two fistular openings in the wall of the jejunum established connection between the intestinal lumen and the described "cavity."

Duodenum.—The first and second portions of the duodenum were moderately distended. Between the plicae, the mucosa exhibited numerous raised nodules and polyps which near their tip measured about 2–3 mm. in diameter (Fig. 8).

From a point about 20 cm. below the pylorus and extending to the jejunal flexure a huge tumour mass had transformed the duodenum into a wide sac (Fig. 3). In this area the internal surface of the duodenum was made rugged by

vegetations of friable tissue which presented a moist and greyish cut-surface, frequently including yellowish specks or wider areas of the same colour. Tissue of this character extended through all layers of the intestinal wall. In some cases the tissues were broken down and only a paper-thin membrane separated the lumen from adjacent structures.

The posterior wall of the duodenum was adherent to nodular masses which were formed as described above by parapancreatic and paraportal lymph nodes being replaced by tumour tissue.

Jejuno-ileum—Mucosa was extremely pale. There were tiny polyps of the kind seen in the first and second portions of the duodenum. In the direction of the ileum the number of polyps became less, and in the ileum they were sparse.

About 20 cm. behind the large tumour, there were two ulcerations, each measuring about 10 cm. in diameter. Within their limit the intestinal wall showed firm thickening, and the floor contained narrow fistules which opened into the abscess cavity.

Colon.—Mucosa was pale ; it presented a few flat elevations which measured between 3 and 5 cm. in diameter. Two small polyps were found near the hepatic flexure and in the transverse colon. The heads of the polyp measured about 4 mm. in diameter and were slightly redder in colour and of the same consistency as the surrounding mucosa.

Histological Examination.

Duodenum.—Medullary carcinoma containing only very sparse stroma. The cells exhibited extreme pleomorphism and typical and atypical mitotic figures were found in abundance. Extensive necrosis was present in many areas.

Jejunum.—In several fragments taken for examination, small polyps were seen, some of them arising from the side of jejunal folds, others lying between the latter. The histological aspects were in no way different from those found in Cases I and II.

Colon.—The pedicle of the polyps was built of submucosa and muscularis mucosae. The mucosa near the tip of the polyps was slightly thicker than in their vicinity. The glands displayed slight irregularities in outline and some of them were moderately dilated. In several glands the goblet cells were replaced by groups of dark epithelia containing eosinophilic cytoplasm. In the vicinity of one of the polyps small foci of thickening of the mucous membrane due to increase of glandular tissue were found.

EXPLANATION OF PLATES.

FIG. 1.—Duodenum with pylorus in Case I. Besides the ulcerated carcinoma, there are several adenomatous polyps (Fig. 6) and in the upper third of the photograph, numerous small polyps (Fig. 4).

FIG. 2.—Case II : Between the large ulcerated carcinoma and pylorus a pedunculated polyp is seen and distally from the carcinoma there are scattered polyps.

FIG. 3.—Case III : Large ulcerated carcinoma. The floor of the ulcer is formed by lumps of lymph-nodes with metastatic carcinoma. Between carcinoma and pylorus there are innumerable small polyps (Fig. 8).

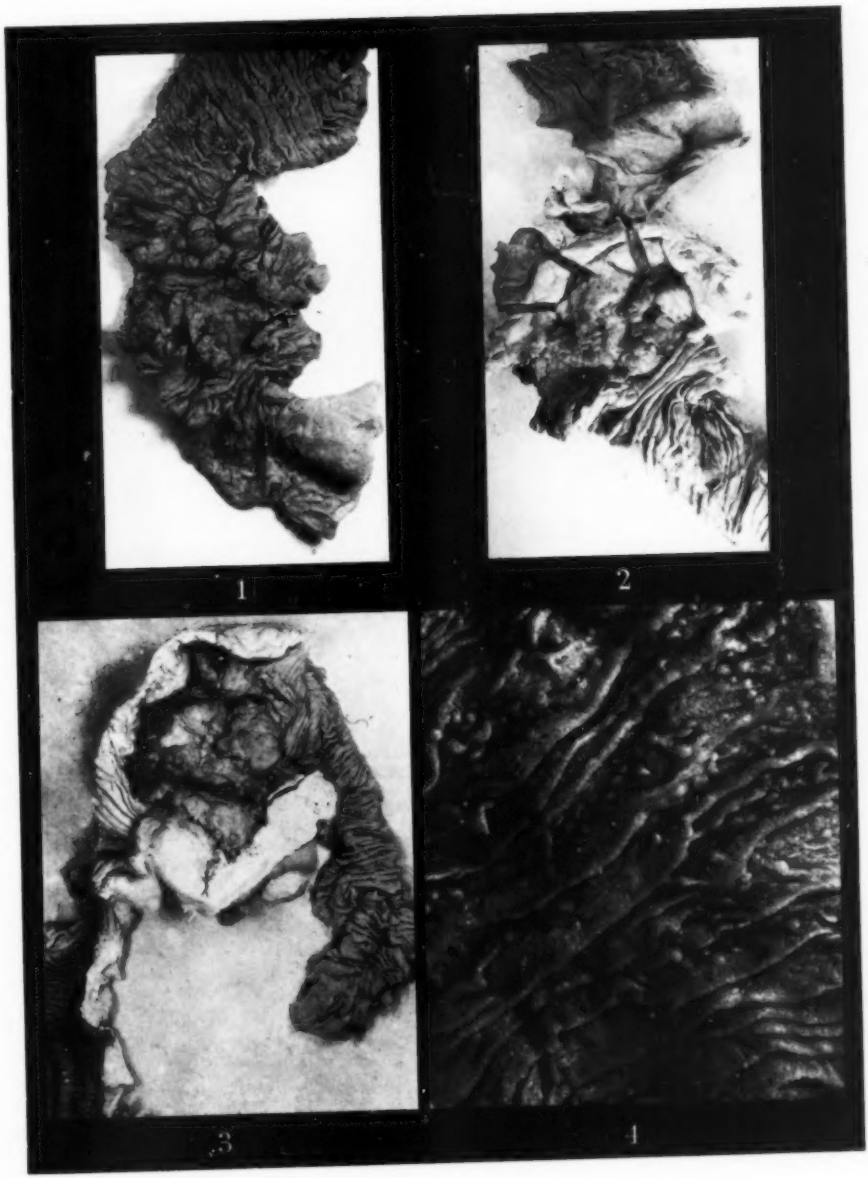
FIG. 4.—Polyposis of duodenum in Case I.

FIG. 5.—Papillary carcinoma (Case I).

FIG. 6.—Adenomatous polyp of duodenum (Case I).

FIG. 7.—Adenomatous polyp of colon (Case I).

FIG. 8.—Polyposis of duodenum in Case III.



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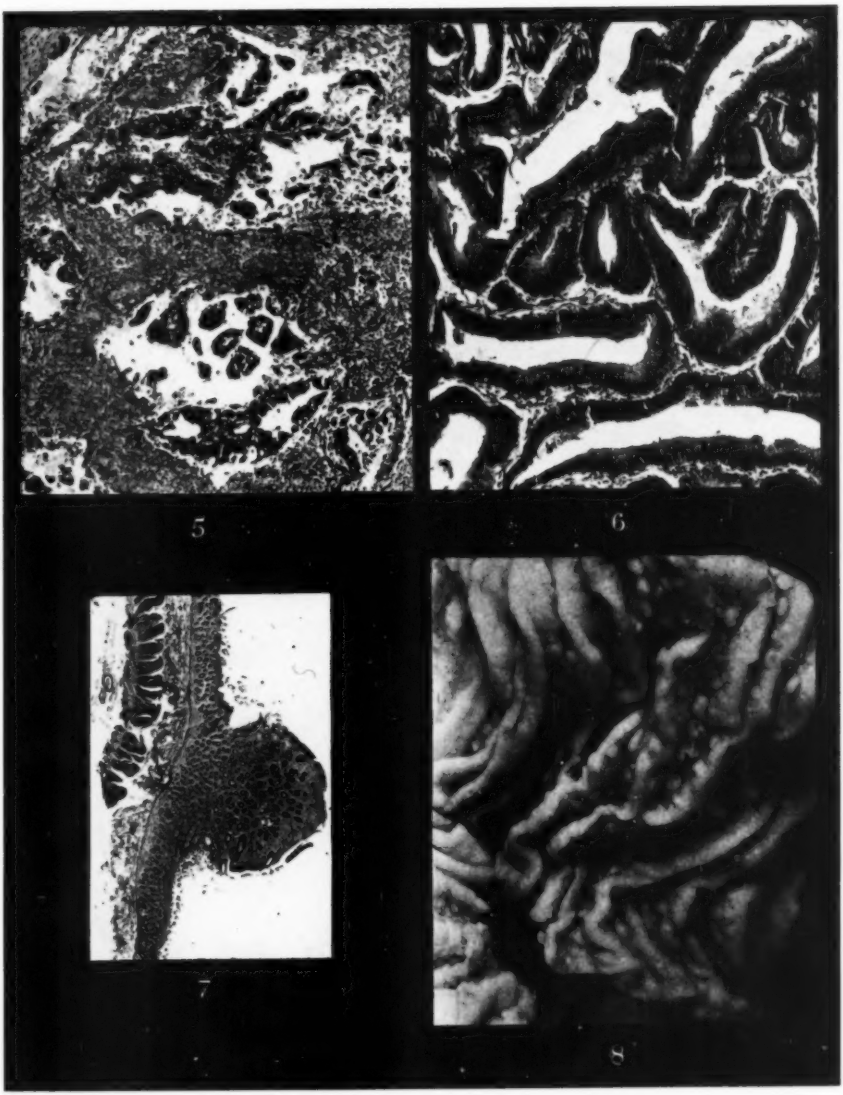

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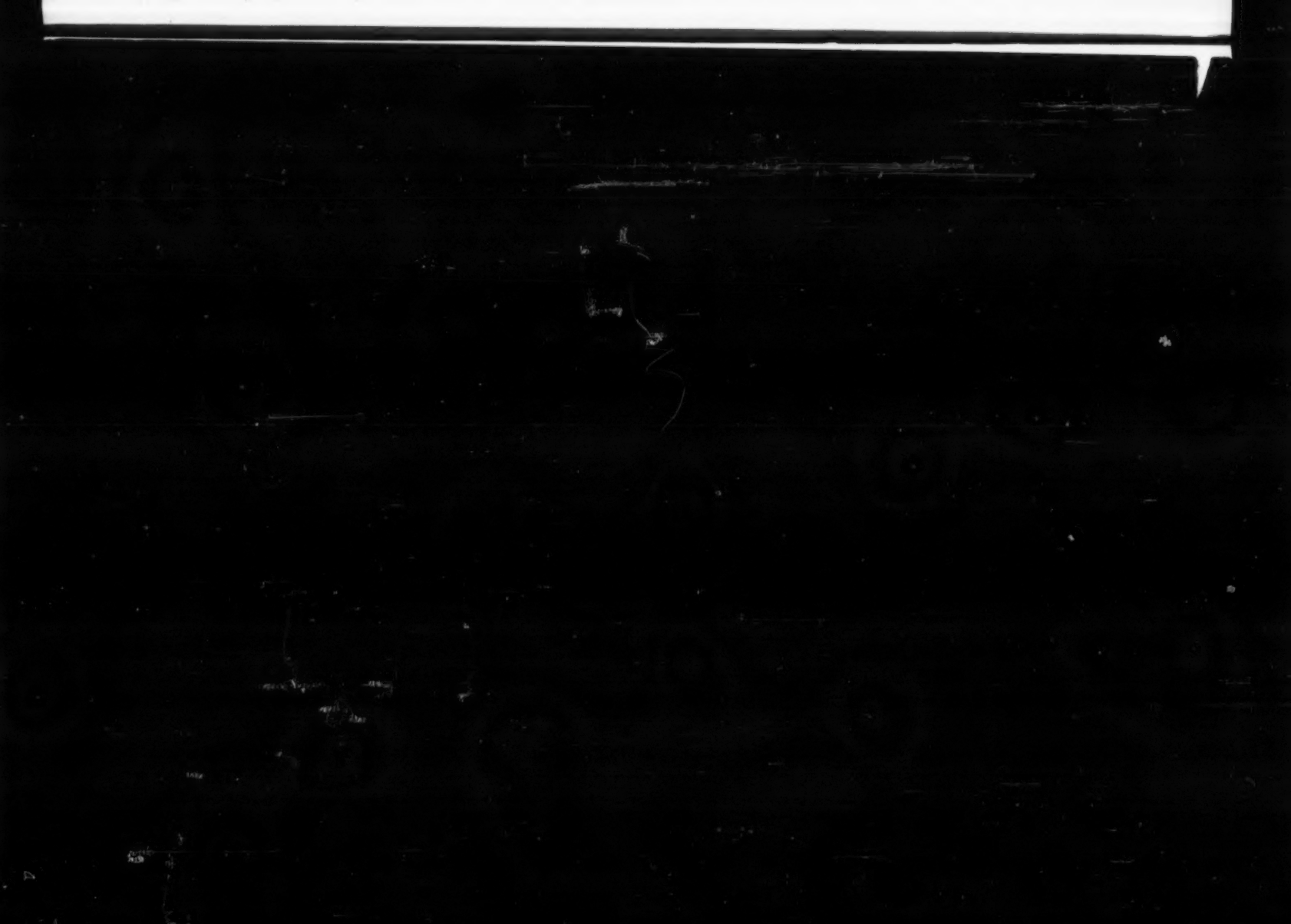

FAMILY HISTORY OF CASES SEEN AT AUTOPSY.

The parents, of Yemenite Jewish stock, were born in the Old City Quarter of Jerusalem and there spent all their life. No previous illnesses were remembered. They married in 1918 and had nine children (Table I). Five children died. Three of them—the first, the third and the fourth—were examined post mortem. The findings in these form the subject of this article. Of the two others, it is known that one died from "toxicosis" in infancy and one from typhoid fever. In these cases, no autopsy was performed.

TABLE I.

Yaacob ♂					Sarah ♀			
Rivkah ♀	Yehuda ♂	Chaim ♂	Itzhak ♂	Eliezer ♂	Rachel ♀	Simha ♂	Moshe ♂	Joseph ♂
b. 1919, d. 1939. Carcinoma duodeni.	b. 1921.	b. 1923, d. 1939. Carcinoma duodeni.	b. 1926, d. 1944. Carcinoma duodeni.	b. 1928.	b. 1929, d. 1929. "Toxicosis in infancy."	b. 1931, d. 1939. Typhoid fever.	b. 1934.	b. 1936.

The following is known concerning the four surviving children:

The now eldest son Yehuda was born in 1921. His previous history contains no data of pertinent interest. In 1944 he underwent an operation for a traumatic lesion of the liver. During laparotomy the operating surgeon, who was aware of our interest in the case, palpated the intestines but found no evidence of a tumour. An X-ray examination of the stomach and duodenum 2 months following the operation likewise failed to reveal pathologic changes.

The three younger boys were kept under observation from birth by the Infant Welfare Centres and the School Hygiene Departments of the Hadassah Medical Organisation. The respective records contain no references to gastro-intestinal disorders.

In 1946 two X-ray examinations of the 20 years, old boy Eliezer were performed. No abnormalities were disclosed by the radiographs.

COMMENT.

A search of the literature failed to reveal any previous report on appearance of duodenal tumour in several members of one family. Only nine cases of duodenal carcinoma during the first and second decade of life could be collected (Table II).

TABLE II.—*Carcinoma Duodeni in Patients below the Age of 20.*

Author.	Year.	Age of patient.
Ewald*	1896	16 years
Bernouilli*	..	18 ,,
Whittier†	1889	Case 1: 17 years
		,, 2: 18 ,,
		,, 3: 18 ,,
Brill	1904	17 years
Oberndorfer	1929	13 ,,
Lieber, Stewart and Lund	1939	15 ,,
Hoffman and Pack (Case 11)	1937	18 ,,

* Quoted from Borrman (1926).
† Quoted from Hoffman and Pack (1937).

There are, however, in the literature several cases in which epithelial tumour in the duodenum was co-existing with multiple benign or malignant tumour in other parts of the gastro-intestinal tract (Table III).

TABLE III.—*Cases in which Epithelial Tumour of the Duodenum Co-existed with Epithelial Tumours in other Portions of the Gastro-intestinal Tract.*

Author.	Age and sex of patient.	Localization of: Benign polyposis.	Malignant tumours.
1. Hauser (1895)*	M. 33 years	Duodenal polyp, polyposis recti et colon	Carcinoma colon.
2. Petrow (1896)†	F. 20 „	Duodenum, jejunum, ileum	..
3. Funkenstein (1904)*	F. 24 „	Duodenum, stomach, colon, rectum	..
4. Döhring (1907)	F. 32 „	Duodenum, stomach, caecum, colon	Four carcinomas in transverse colon and rectum.
5. Versé (1908)*	F. 32 „	Adenomas in duodenum, multiple polyps in caecum, colon, rectum	..
6. Wechselmann (1910) (case 1)	F. 62 „	Duodenum, jejunum, colon	..
7. Maurice (1913) (quoted after Wirth)	F. 23 „	Polyps in duodenum and jejunum	..
8. Nagel (1925)	?	Duodenum, jejunum	Malignant adenoma of duodenum, carcinoma ventriculi.
9. Markus (1933)	F. 40 years	Benign polyposis of stomach	Malignant adenoma of duodenum.
10. Cassidy and Maschia (1934)	F. 29 „	Polyps in duodenum and jejunum	Several malignant polyps.

* Quoted from Golden.
† Quoted from Döhring.

The comparison of these cases and of our own with generalized polyposis of the intestines is of interest. In this condition benign polyps exhibit a definite tendency to malignant change. The disease is hereditary and affects patients of all age groups, including the lowest. Since generalized polyposis is known to involve predominantly the large intestine, it is not infrequently mentioned in textbooks and articles under the name "polyposis or adenomatosis of the colon." However, in rare cases, the disease was found to be prevalent in the small intestine. Besides the instances mentioned in Table III, others of this variant have been described by Lubarsch in 1888 (Döhring 1907), Schwytzer (1924), Roan (1932), Haggard and Floyd (1935), Ravitch (1948).

Some pathologists have taken the view that generalized polyposis is an entity which since it causes characteristic severe dysentery-like symptoms and malignant change in a high percentage of cases, should be separated from that represented by cases in which only few polyps are formed (Oberndoerfer, 1929; Lockhart-Mummery, 1934; Hullsiek, 1928). On this view our Case III and probably also Case I would certainly have to be comprehended under generalized polyposis, but Case II, which presented only scattered small polyps besides the carcinoma of the duodenum, would not belong to this group.

Recent experience has tended, however, to detract from the significance of the nosologic separation of "generalized polyposis" from "single or multiple polyps." Several authors were able to show that lesions of this type need not

be congenital and can arise at various times over a protracted period of life (McKenney, 1936; Lockhart-Mummery and Dukes, 1928; Haggard and Floyd, 1935). It has become evident that differences in the speed of progress of the disease can delay its clinical manifestation even to the seventh and eighth decade of life (Kaufmann, 1931; Coffey and Bargen, 1939). Considering the high incidence of malignant change in intestinal polyposis, an estimated 62 per cent to almost 100 per cent of the cases (Lockhart-Mummery and Dukes, 1928; Coffey and Bargen, 1939), it is to be expected that in certain cases patients may die from intestinal carcinoma before the polyposis has had time to generalize. This possibility is evident also from genealogical tables of the affected families, in which cases of generalized polyposis, single polyps and carcinoma of the intestines without any polyps occur randomly (Jüngling, 1928).

It may be inferred from the foregoing that the hereditary and familial character of the disease is the decisive factor in the diagnosis. The designation of all three of our cases as generalized polyposis therefore seems to be justified.

The observations reported in this article are relevant to the understanding of factors which govern the manifestation of malignancy in intestinal polyposis. Several authors have assumed a casual relationship between the appearance of polyps and carcinoma in any given case. They believed that the chance for malignant transformation is increased when there is a numerical increase of polyps (Lockhart-Mummery, 1934; Hurst, 1939). Other investigators have related the sites of predilection of intestinal carcinoma to the greater irritation of the mucosa in the areas of physiological faecal stasis, viz. the rectum, sigmoid colon, the region of the colic flexures and near the ileo-caecal valves (Oberndoerfer, 1929).

On the other hand, the published histories of families in which malignant neoplasm appeared in several members favour the assumption that the site of malignant manifestation is determined by one specific genetic agent and that the histological character and also the age of onset of the malignant change are determined independently (Macklin, 1935, 1938). The action of a genetic agent for the site of the malignant transformation in previously benign polyposis cannot be proved beyond doubt in a localization, e.g. rectum or colon, in which the incidence of intestinal carcinoma is high even in non-hereditary cases. However, in view of the extreme rarity of the development of carcinoma of the duodenum in the earlier decades of life, the operation in our cases of all three mentioned factors appears to be very probable.

SUMMARY.

Three instances of carcinoma of the duodenum with concomitant polyposis of the small intestine and single polyps of the colon are reported.

The patients were siblings, two brothers and a sister, 16, 18 and 19 years of age respectively.

The literature on duodenal carcinoma in young patients is reviewed. Possible implications of this observation for the understanding of malignant transformation in intestinal polyposis are discussed.

Permission to use the clinical records was kindly given by Dr. M. Rachmilewitz and Professor J. Kleeberg, heads of the Departments of Medicine of the Hadassah University Hospital in Jerusalem.

REFERENCES.

BORRMANN, R.—(1926) ' Handb. Spez. Pathol. Anat.', Vol. IV, pt. 1. Berlin.
BRILL, N. E.—(1904) *Amer. J. med. Sci.*, **128**, 824.
CASSIDY, J. M., AND MACCHIA, B. J.—(1934) *Amer. J. digest. Dis.*, **1**, 755.
COFFEY, R. J., AND BARGEN, J. A.—(1939) *Surg. Gynec. Obstet.*, **69**, 136.
DÖHRING, H.—(1907) *Arch. klin. Chir.*, **83**, 194.
GOLDEN, R.—(1928) *Amer. J. Roentgenol.*, **20**, 405.
HAGGARD, W. D., AND FLOYD, W. O.—(1935) *Amer. J. Surg.*, **31**, 428.
HOFFMAN, W. J., AND PACK, G. T.—(1937) *Arch. Surg.*, **35**, 11.
HULLSIEK, H. E.—(1928) *Surg. Gynec. Obstet.*, **47**, 346.
HURST, A.—(1939) *Lancet*, i, 553, 621.
JÜNGLING, O.—(1928) *Beitr. klin. Chir.*, **143**, 476.
KAUFMANN, E.—(1931) ' Lehrbuch der Speziellen Pathologischen Anatomie,' 9th ed. Berlin : de Gruyter.
LIEBER, M. M., STEWART, H. L., AND LUND, H.—(1939) *Ann. Surg.*, **109**, 383.
LOCKHART-MUMMERY, J. P.—(1934) *Ibid.*, **99**, 178.
Idem, AND DUKES, C.—(1928) *Surg. Gynec. Obstet.*, **46**, 591.
MACKLIN, M. T.—(1935) *Medicine*, **14**, 1.—(1938) Univ. Wisconsin, Symposium on Cancer, p. 32.
MCKENNEY, D. C.—(1936) *J. Amer. med. Ass.*, **107**, 1871.
MARKUS, H.—(1933) *Klin. Wschr.*, **12**, 617.
NAGEL, G. W.—(1925) *Arch. Surg.*, **11**, 529.
OBERNDOERFER, S.—(1929) ' Handb. Spez. Pathol. Anat.', Vol. IV, pt. 3. Berlin.
RAVITCH, M. M.—(1948) *Ann. Surg.*, **128**, 283.
ROAN, O.—(1932) *Tex. St. J. Med.*, **27**, 782.
SCHWYTZER, R.—(1924) *Schweiz. med. Wschr.*, **5**, 869.
WECHSELMANN, L.—(1910) *Beitr. klin. Chir.*, **70**, 855.
WIRTH, W.—(1913) Inaug.-Dissertation, Berlin.

CULTURE OF HUMAN LEUKAEMIC BLOOD CELLS *IN VITRO*; SOME EFFECTS OF X-RAYS.

F. W. GUNZ.*

From the Department of Radiotherapeutics, University of Cambridge.

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IN previous publications (Gunz, 1948*a*, *b*) a technique of cultivating human leukaemic leucocytes *in vitro* was described, and the quantitative and cytological findings in untreated cultures made by it were analysed. The present paper is intended to set out results obtained by the application of this technique to the investigation of the action of X-rays. Two groups of experiments are reported. In the first, leucocytes were directly irradiated *in vitro*. In the second, they were explanted after therapeutic irradiation of the patient. It was hoped that, by thus comparing the effects, seen in identical conditions, of irradiation *in vitro* and *in vivo*, some indications might be found of the mechanism by which X-rays produce their action in patients suffering from leukaemia.

Blood cells have been irradiated *in vitro* by a number of investigators, beginning with Linser and Helber (1905), who, in the course of extensive irradiation

* Saltwell Research Student, Royal College of Physicians of London.

experiments on living animals, also exposed to X-rays suspensions of normal blood cells and examined them in hanging drops, finding, as a result of the application of large doses, a diminution in the number of cells, and alterations in the appearance of both nuclei and cytoplasm. Similar experiments were made with normal animal blood by Jolly and Lacassagne (1923), Lacassagne and Gricouroff (1927), and Ono (1929); with normal human blood by Neumann (1924) and Jansson (1927); with animal lymph nodes by Stenstrom and King (1934), and animal bone marrow by Meldolesi and Giusti (1934) and Gregori (1939). Certain features are common to this group of papers: in all of them the authors assessed the effects of radiations (X-rays or from radon) by noting either changes in the appearance of resting cells—especially cell destruction—or in the degree of cellular emigration; and, in order to produce such changes, they had to administer very large doses. Thus, Neumann found abnormal appearances attributable to X-rays in only two out of twelve bloods after 60 H (equivalent to about 12,000 r), while no impairment of the outgrowth of rabbit bone marrow cultures was noted by Gregori after doses of X-rays of up to 10,000 r. However, neither immediate destruction nor impairment of motility is a sensitive index of cellular damage. Spear (1935) states that the dose of X-rays required for a direct lethal action on cultured cells may exceed that necessary for interference with mitosis by a factor of 3000.

Another group of authors used doses of X-rays partly or wholly within the therapeutic range. Here must be mentioned especially a collection of papers from Osgood's laboratory (Osgood and Bracher, 1939; Osgood, 1940; Osgood, 1942; Osgood, Aebersold, Erf and Packham, 1942), in which cells from normal human bone marrow and leukaemic blood were irradiated in liquid cultures and total and differential counts were done on them. It was found that with doses of X-rays as low as 50 r there was a fall in the number of lymphocytes and early myelocytes, beginning during the first 24 hours and continuing during the rest of the experiments (seven days). An increase in the dose produced a steeper decline in these cells, the effect increasing approximately with the square root of the dose given. No changes in the morphology of irradiated cells were seen. The effect could not be produced by incubating unirradiated cells in plasma which had been irradiated *in vitro*. Leukaemic promyelocytes were more sensitive than normal ones. No difference was noted between the effects of the same doses of X-rays given at 200 or 1000 KV, 1 n of neutrons produced the same results as 4 r of X-rays given at 200 or 1000 KV, and 1 μc./ml./24 hours of P^{32} the same as 35 r. Osgood suggested that ionizing radiations acted probably by inhibiting mitosis. He did not, however, determine the mitotic rates in his cultures, because of the small number of dividing cells present.

Osgood's findings may be compared with those of Schrek (1946*a*, *b*), who irradiated suspensions of thymus and spleen lymphocytes, and of bone marrow cells (rabbit), and of normal and leukaemic human blood cells in "phosphate Ringer solution" with and without homologous serum, with doses of X-rays of 20–4000 r. Using as a criterion of survival a failure of cells to stain with a 1:1000 solution of eosin in Tyrode solution, Schrek found exposure to X-rays to cause a decreased survival time *in vitro* in lymphocytes from the thymus, normal blood and four of five bloods from patients with chronic lymphatic leukaemia. Such an effect was noted after a dose of 50 r, and increased with an increase in the dose up to 1000 r, remaining approximately constant at higher doses. No

effect was, however, demonstrable in rabbit marrow cells, or in the bloods from one patient with chronic lymphatic or seven patients with chronic myeloid leukaemia. The author, discussing possible reasons for this lack of effect, attributes it to an absence of dividing myeloid cells in his preparations, X-rays leaving resting myeloid cells unaffected.

Interference with mitosis, as a result of X-irradiation, was definitely demonstrated by Rachmilewitz, Rosin, Goldhaber and Doljanski (1945, 1947) in experiments with fragments of explanted rabbit bone marrow. Following the application of 250–1000 r there was a depression of mitosis lasting for two to four hours, with subsequent recovery. With higher doses the depression was more profound, and recovery incomplete. Many mitotic abnormalities were caused, and the marrow became hypocellular, or almost completely aplastic, according to the size of the dose given.

The observations in the literature may be summarized by stating that X-rays have been found to damage blood and haemopoietic cells, especially lymphocytes and all classes of immature cells, when applied directly *in vitro*, and that disturbances of mitosis appear to be among the earliest signs of injury in cells so exposed. The effect of X-rays on mitosis in human cells has, however, not been investigated.

MATERIAL AND TECHNIQUE.

The present investigations were made on leucocytes obtained from the venous blood of eight patients with chronic myeloid leukaemia, which is particularly suitable for the purpose. The course of the disease in all patients was typical of that usually observed. Two patients had not received any X-ray treatment when first seen ; the others had had various amounts of treatment in the past.

The technique of culture was that previously described (Gunz, 1948*a*). In brief, it consisted of the distribution of small equal quantities of leukaemic leucocytes, suspended in the culture medium, over a series of culture tubes, and the withdrawal of some of the tubes for counting after varying periods of incubation. At these times total and differential counts were made, and the total number and phase distribution of mitoses determined. The culture medium used consisted either of 50 per cent dried, reconstituted human plasma, with the addition of 5 per cent rat embryo extract, or of 50 per cent fresh homologous human serum, both diluted in Ringer solution. The duration of experiments was two days, and counts were done at 8, 24 and 48 hours, unless otherwise stated.

The technique of irradiation was as follows : after the cultures had been set up, they were incubated at 37° for about 15 minutes, to give them time to reach body temperature. In some experiments some of the tubes were also incubated for longer periods before irradiation. They were then removed to the X-ray room, shaken and irradiated at once. No special precautions were taken to keep their temperature constant during irradiation, but as this (with the exception of two experiments in which 10,000 r were given) required at the most 97 seconds, and was carried out in a warm room, no significant change of temperature should have taken place. Following irradiation, the tubes were immediately returned to the incubator.

The irradiations were carried out by means of a G.E. Maximar " 220 " apparatus, and the following radiological factors were employed : 220 KV,

15 mA, and 1·0 mm. Al filter. The tubes were exposed at two fixed distances (approximately 21 and 81 cm. from the target), the dose rates, measured in air, at these points being 622 and 62r /min. respectively. The actual dose rate to which cells were exposed depended on three factors: the dose rate in air, the absorption and scatter due to the glass, and the absorption and scatter due to the fluid. The dose rate in air was measured by means of a Victoreen dose meter in the normal way; it was found that when the chamber of the dose meter was inserted into empty culture tubes, the average decrease in the dose rate was 6·5 per cent. This enabled a correction to be made for the glass of the tubes. The volume of the liquid being only of the order of 1 or 2 ml., the correction for the absorption and scatter due to it would be less than 5 per cent, and was ignored in estimating the dose rate. Dose measurements were kindly made by Mr. J. L. Haybittle, of the Radiotherapeutic Centre, Addenbrooke's Hospital, Cambridge.

RESULTS.

A. *Irradiation* in vitro.

The doses given were 10, 50, 100, 1000, and 10,000 r. No effect was noted with 10 and 50 r. The results of irradiation with 100, 1000, and 10,000 r are set out in Tables I–VI.

If the contents of Tables I–VI be summarized and analysed, we obtain the following findings:

Immature cells.

With a dose of 100 r the ratio, at 8 hours, of immature cells in irradiated to those in control cultures varied from 65–113 per cent. In five experiments it was greater, and in seven experiments less than 100 per cent. A significance test (Student's t-test) was employed. In the analyses of immature cell counts percentages were first converted to logarithms, in order to obtain a more normal distribution of the series. The actual formula used was $t = \frac{\bar{x} - m}{s_{\bar{x}}}$, where $\bar{x}$ is the arithmetic mean of the series, s_x its standard error, and $m = \log_{10}100 = 2$. This gave a value of $t = 0{\cdot}97$, which is not significant. At 24 hours the ratio varied from 71 to 111 per cent. In four experiments it was greater, in one equal to, and in seven less than 100 per cent. A significance test gave a value of $t = 2{\cdot}05$; p is greater than 5 per cent., i.e. the difference is not significant. At 48 hours the ratio varied from 48 to 112 per cent. In one experiment it was greater, and in ten experiments less than 100 per cent. From this $t = 5{\cdot}25$, and p is less than 0·001. There was, therefore, a highly significant reduction in the number of immature cells in cultures 48 hours after irradiation with 100 r.

With a dose of 1000 r the ratio, at 8 hours, of immature cells in irradiated to those in control cultures varied from 76 to 111 per cent. In three experiments it was greater, and in three experiments less than 100 per cent. A significance test gave a value of $t = 1{\cdot}11$, which is not significant. At 24 and 48 hours the ratio was less than 100 per cent in all experiments. t-tests gave values of 4·56 and 5·52 respectively, both of which correspond to $p =$ less than 0·01. There

was therefore a highly significant reduction in the number of immature cells in cultures 24 and 48 hours after irradiation with 1000 r. Results with 10,000 r were similar.

TABLE I.—*Effect of Irradiation with* 100 *r on Immature Cell Counts of Cultures of Human Leucocytes (Chronic Myeloid Leukaemia).*

Case No.	Date.	Immature cells (% of initial count). 8 hours. Cont.	Irr.	Irr./Cont. (%)	24 hours. Cont.	Irr.	Irr./Cont. (%)	48 hours. Cont.	Irr.	Irr./Cont. (%)
1	10.iii.48	105	100	95	91	96	105	96	108	112
1	22.iii.48	87	94	108	72	62	86	63	43	68
2	13.iv.48	72	82	113	63	63	100	54	26	48
3	5.v.48	89	86	96	70	68	97	58	38	65
4	7.v.48	110	119	108	86	61	71	66	48	73
5	12.v.48	97	87	90	94	78	83	82	62	76
6	26.vii.48	58	63	108	47	43	91	27	23	85
7	15.ix.48	120	117	97	80	84	105	59	46	78
1	22.ix.48	87	57	65	50	42	84	29	17	59
8	6.x.48	100	93	93	86	72	84	72	51	71
8	20.x.48	86	77	89	43	48	111	—	—	—
8	2.ii.49	91	92	101	51	52	102	49	33	67

TABLE II.—*Effect of Irradiation with* 100 *r on Mitotic Count of Cultures of Human Leucocytes (Chronic Myeloid Leukaemia).*

Case No.	Date.	Mitotic cells (per 1000 immature cells). 8 hours. Cont.	Irr.	Irr./Cont. (%)	24 hours. Cont.	Irr.	Irr./Cont. (%)	48 hours. Cont.	Irr.	Irr./Cont. (%)
1	10.iii.48	5·5	0	0	3·5	1·5	42	2	3·5	175
1	22.iii.48	3·5	0	0	3	4·5	150	0	1	∞
2	13.iv.48	8·7	0·3	3·4	5·7	4·3	75	0·7	2·3	328
3	5.v.48	1	0	0	2·3	1·3	56	0	1·3	∞
4	7.v.48	4	0	0	4	3·3	82	2·3	4	174
5	12.v.48	2·3	0	0	2·7	1·7	62	0·7	2·3	330
6	26.vii.48	12·7	5·3	42	31	27·3	88	91·3	64·7	70
7	15.ix.48	5	0	0	23·5	13	55	34	35	103
1	22.ix.48	2	0·7	35	6·7	15	220	15·7	8·7	55
8	6.x.48	13	3	23	17	20·3	119	10·3	15·3	148
8	20.x.48	8	4	50	15·3	8·7	57	—	—	—
8	3.xi.48	4·7	0	0	8·7	8	92	16	12·5	78
8	2.ii.49	13·3	4·7	35	21	16·3	77	49	27·3	56

TABLE III.—*Effect of Irradiation with* 1000 *r on Immature Cell Counts of Cultures of Human Leucocytes (Chronic Myeloid Leukaemia).*

Case No.	Date.	Immature cells (% of initial count). 8 hours. Cont.	Irr.	Irr./Cont. (%)	24 hours. Cont.	Irr.	Irr./Cont. (%)	48 hours. Cont.	Irr.	Irr./Cont. (%)
1	10.iii.48	105	117	111	91	83	91	96	65	67
1	22.iii.48	87	91	104	72	52	72	63	33	52
2	13.iv.48	72	76	105	63	48	76	54	21	39
5	12.v.48	97	80	82	94	76	81	82	44	53
8	6.x.48	100	76	76	86	45	52	72	22	30
8	8.x.48	93	81	87	102	57	56	—	—	—
8	21.x.48	—	—	—	92	46	50	—	—	—

TABLE IV.—*Effect of Irradiation with* 10,000 *r on Immature Cell Counts of Cultures of Human Leucocytes (Chronic Myeloid Leukaemia).*

Case No.	Date.	Immature cells (% of initial count). 8 hours. Cont.	Irr.	Irr./Cont. (%)	24 hours. Cont.	Irr.	Irr./Cont. (%)	48 hours. Cont.	Irr.	Irr./Cont. (%)
1	10.iii.48	105	128	122	91	61	68	96	29	30
1	22.iii.48	87	80	91	72	39	54	—	—	—

TABLE V.—*Effect of Irradiation with* 1000 *r on Mitotic Count of Cultures of Human Leucocytes (Chronic Myeloid Leukaemia).*

Case No.	Date.	Mitotic cells (per 1000 immature cells). 8 hours. Cont.	Irr.	Irr./Cont. (%)	24 hours. Cont.	Irr.	Irr./Cont. (%)	48 hours. Cont.	Irr.	Irr./Cont. (%)
1	10.iii.48	5·5	0	0	3·5	1	28	2	0	0
1	22.iii.48	3·5	0	0	3	0	0	0	0	0
2	13.iv.48	8·7	0	0	5·7	0	0	0·7	0	0
5	12.v.48	2·3	0·3	13	2·7	0	0	0·7	0	0
8	6.x.48	13	1	8	17	0·3	2	10·3	3·3	32
8	8.x.48	14·5	1·5	10	11·5	0	0	—	—	—
8	21.x.48	—	—	—	8·5	0·5	6	—	—	—
8	3.xi.48	4·7	0	0	8·7	0	0	16	0	0

TABLE VI.—*Effect of Irradiation with* 10,000 *r on Mitotic Count of Cultures of Human Leucocytes (Chronic Myeloid Leukaemia).*

Case No.	Date.	Mitotic cells (per 1000 immature cells). 8 hours. Cont.	Irr.	Irr./Cont. (%)	24 hours. Cont.	Irr.	Irr./Cont. (%)	48 hours. Cont.	Irr.	Irr./Cont. (%)
1	10.iii.48	5·5	0	0	3·5	0	0	2	0	0
1	22.iii.48	3·5	0	0	3	0	0	—	—	—

Mitoses.

With a dose of 100 r the ratio, at 8 hours, of the number of cells in mitosis in irradiated to those in control cultures varied from 0 to 50 per cent; in 7 out of 13 irradiated cultures no mitoses were present 8 hours after irradiation. The chance of 13 decreases in the number of mitoses occurring accidentally in 13 experiments is 1/4096. The reduction in the mitotic count 8 hours after 100 r is therefore clearly significant. At 24 hours the ratio varied from 42 to 220 per cent. In 3 experiments it was greater, and in 10 experiments less than 100 per cent. A significance test gave a value of $t = 1{\cdot}64$, which is not significant. No analysis was carried out for the 48-hour values.

With a dose of 1000 r the ratio was less than 100 per cent in all experiments, and at all times. No mitoses were present in irradiated cultures in 4 out of 7 experiments at 8 hours, in 5 out of 8 experiments at 24 hours, and in 4 out of 6 experiments at 48 hours. The highest percentage of mitoses present was 32. There was, therefore, clearly a highly significant reduction in the number of

mitoses at all times after irradiation with 1000 r (the combined p for all three times is less than 0·001). The results with 10,000 r were similar.

It must be emphasized that these experiments were performed with varying culture media, and that the cells in some of them came from untreated, and in others from previously treated patients. This explains many of the numerical differences between individual experiments, particularly in the mitotic counts. However, in spite of the non-homogeneity of the material, the results show clearly the following points:

Irradiation of cultures of leucocytes from chronic myeloid leukaemias with 100 r produced a significant reduction in the number of dividing cells for at least 8 hours. Mitosis then recovered, and did not differ significantly from controls at 24 hours or later. The temporary hold-up in mitosis did not cause a reduction in the number of immature cells until 48 hours after irradiation.

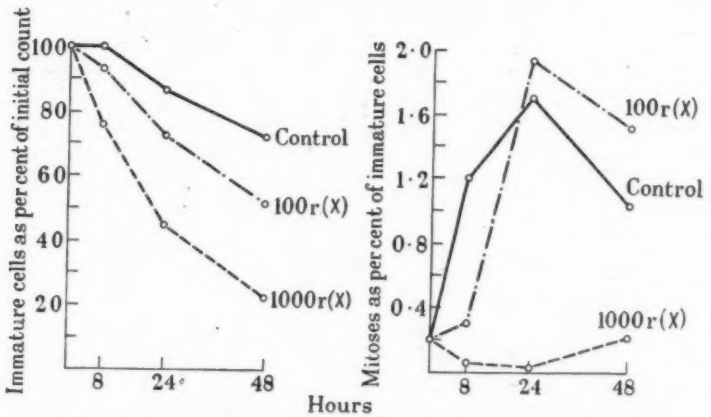


FIG. 1.—*In vitro* irradiation of leucocytes (chronic myeloid leukaemia).

Irradiation with 1000 and 10,000 r produced a more marked depression of mitosis at 8 hours, and this did not recover subsequently. The result was a significant falling-off in the number of immature cells from 24 hours onwards. Mature granular cells remained equally numerous in irradiated and control cultures. Fig. 1 illustrates the findings in a typical experiment with 100 and 1000 r.

Effect of varying the dose rate.

The dose rate was varied by changing the target distance. In two experiments 100 r was given both at 62 and 622 r/min. No significant difference was noted as a result of changing the dose rate (Fig. 2).

Appearances in stained films.

In the experiments so far reported the examination of stained films made from irradiated cultures did not reveal abnormalities, either of resting or dividing cells, in greater numbers than were present in films of control cultures. In

particular, there was no evidence that X-rays acted by damaging resting cells. As regards mitotic abnormalities, the material was not favourable for the discovery of minor degrees, for the following reasons: some abnormalities, like chromosome aberrations and bridges, and polyploidy are not uncommon in control cultures (Gunz, 1948*b*). Further, pyknotic metaphases become increasingly common in the later stages of all cultures, especially when fresh serum is used. Finally, with the technique of smearing and staining used, good instantaneous fixation of cells is difficult, and hence some clumping of the numerous small chromosomes is almost inevitable; for this reason, such abnormalities as chromosome breaks may be overlooked, even if present. Consequently, only a very pronounced increase in the number of mitotic abnormalities would have been noticeable. When, therefore, we attribute some effects of irradiation to mitotic inhibition, we cannot exclude the possibility that they were in part caused by the production of mitotic abnormalities.

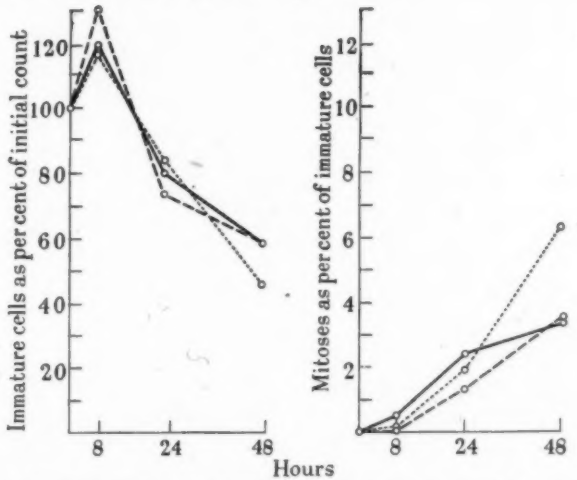


FIG. 2.—Effect of 100 r (X) given at two different dose rates on human leucocytes *in vitro* (chronic myeloid leukaemia).

———— Control.
– – – – – – 100 r (62 r/minute).
. . . . . 100 r (622 r/minute).

There is, however, a further reason in explanation of the apparent absence of abnormal mitoses in these cultures. This is the fact that they had all been irradiated very shortly after their inception. At that stage there were practically no dividing cells present, and the vast majority of all immature leucocytes in the cultures were truly "resting." Only after the lapse of several hours could any considerable numbers of them be expected to be approaching or entering mitosis. It is, however, well known that chromosomes are particularly liable to damage from radiation in the late pre-mitotic or early mitotic stages (Lea, 1946). The following experiments were made in an attempt to irradiate cells at such a moment of increased sensitivity to X-rays, and to determine whether chromosome abnormalities could thereby be induced.

Effect of varying the time of irradiation.

In two experiments different groups of tubes were irradiated with 100 r at 0, 2 and 4 hours from the time of setting up the cultures. The results were similar in both experiments; those in one are shown in Fig. 3, from which it is clear that no significant difference in the mitotic rate or the number of immature cells was produced as a result of varying the time at which the cultures were irradiated by up to 4 hours; neither was the number of mitotic abnormalities in stained films altered.

In another group of three experiments cultures were irradiated with 100 or 1000 r 7 hours after they had been set up, and counts and films were made at frequent intervals after irradiation. It was known that at 7 hours, appreciable numbers of cells would be entering mitosis. The results of one such experiment are shown in Fig. 4. This indicates that with 100 r, mitoses began to decline

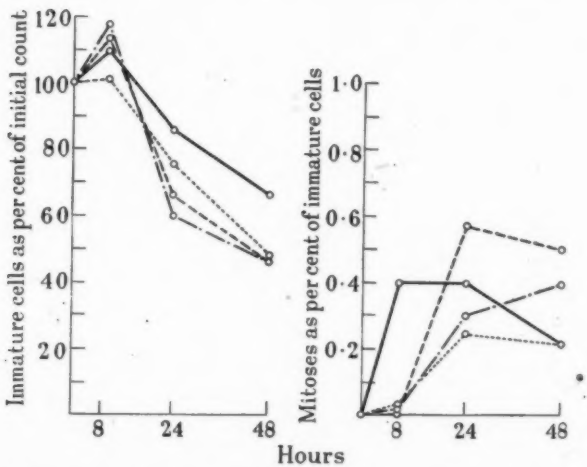


FIG. 3.—Effect of irradiation given at different times on human leucocytes *in vitro* (chronic myeloid leukaemia).

———— Control.
— · — · — 100 r at start.
· · · · · · · · 100 r at 2 hours.
– – – – – – 100 r at 4 hours.

soon after irradiation, but that recovery set in after a few hours, although it was not yet complete at 20½ hours (13½ hours after irradiation). With 1000 r mitoses only began to fall 3 hours after irradiation—a delay accounted for by a notable accumulation of pyknotic metaphases. However, the fall in the number of mitoses, once under way, continued, until, at 20½ hours, practically no mitoses were present. There was also a fall in the number of immature cells.

During the 4 hours following irradiation with 100 and 1000 r, the mitotic rate was falling; however, some cells were found in all stages of normal mitosis. This fact suggests that some cells which, at the time of irradiation, had reached any of the clearly recognizable stages of mitosis—i.e. probably from late prophase

onwards—continued their division in a normal way. Others, however, were clearly held up when 1000 r were given, as shown by a large number of pyknotic metaphases, as well as of ana- and telophases in which daughter nuclei failed to separate completely.

It was interesting, moreover, that 3 hours after irradiation with 1000, but not with 100 r, there appeared a new kind of abnormal cell, present only in very small numbers (less than 1 per cent of immature cells) in controls; these cells became rapidly more numerous, reaching more than 10 per cent of the immature cells 4 hours after irradiation, and then declined again. Few were found at 20½ hours. Some of these abnormal cells are pictured in Fig. 5–14. The fact

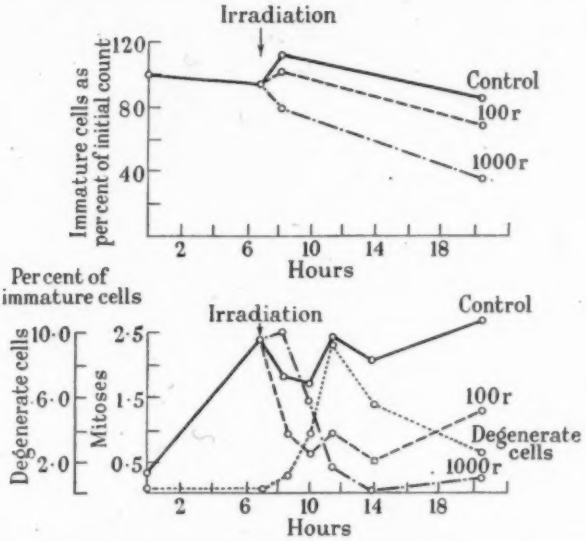


FIG. 4.—Effect of X-rays on leukaemic leucocytes *in vitro*. Irradiation 7 hours after explantation.

that they only appeared when cultures were irradiated at a time when many mitoses were present, suggests that they arose from dividing cells, and it is likely that they originated in early prophase. The figures are arranged so as to make clear the suggested way in which they developed. Fig. 5 shows a normal resting myelocyte; Fig. 6 what is probably a very early prophase; in Fig. 7 the chromosomes are distinct; in Fig. 8 some of them have become clumped; Fig. 9 shows a stage at which the clumping has progressed considerably; in Fig. 10 and 11 some individual chromosomes are still visible, but most of the chromatin material has become aggregated to a structureless mass. Fig. 12, 13 and 14 indicate the end of the process; a progressive breaking-up of the nucleus, and finally of the whole cell. No sign of chromosome fragmentation was found in these or other cells. However, as already mentioned, the material is unsuitable for precise chromosome analyses.

B. *Observation* in vitro *of Cells Irradiated* in vivo.

With two patients it was possible to make repeated cultures before, and at varying intervals after therapeutic irradiation. Results were identical with both. Table VII correlates the findings in the peripheral blood of one patient with the mitotic counts in 8-hour cultures made before, during, immediately and 3 and 5 months after a course of X-ray treatment to the spleen totalling 600 r.

TABLE VII.

Date.	Leucocytes per c.mm. peripheral blood. Total.	Immature.	Mitoses per 1000 immature cells in 8-hour cultures.	X-rays.
6.x.48	400,000	220,000	12	None.
20.x.48	222,000	66,000	8	300 r.
3.xi.48	55,000	8,000	4.7	600 r.
2.ii.49	104,000	34,000	12	No further treatment.
6.iv.49	233,000	77,000	18	

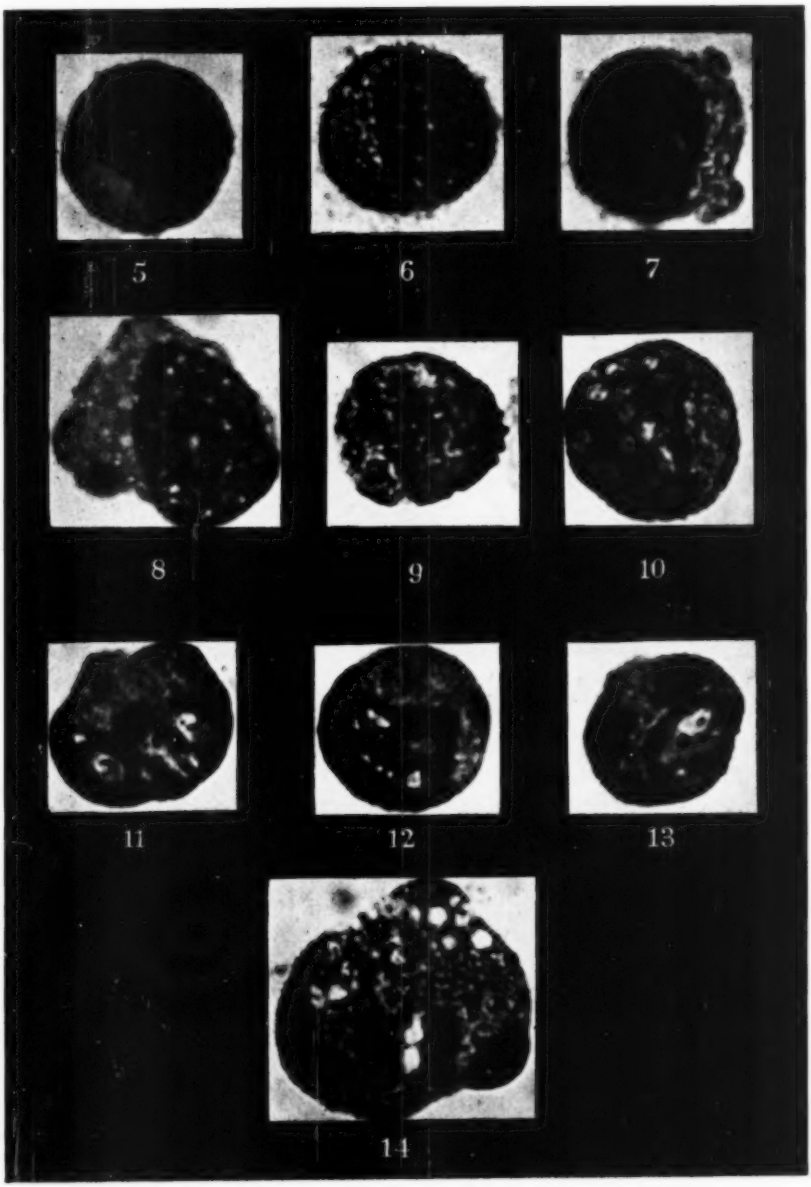
The mitotic counts from the first 3 cultures are also shown graphically in Fig. 15. It would seem that the findings at 8 and 24 hours are most likely to reflect the character of the cells themselves; later on, artefacts due to the composition of the medium become superadded. Taking only the 8-hour counts into consideration, there was a good correlation between the patient's blood count and the number of mitoses in cultures. As the number of total and immature leucocytes fell under treatment, the mitotic activity was decreased in cultures. When, after an interval without treatment, the blood count rose again, cells divided more readily in cultures. In general, mitotic counts were higher in untreated or not recently treated patients than in treated ones (an exception was formed by patients in the terminal stages of leukaemia, in whom mitotic counts were low). It thus appears that therapeutic irradiation induced changes in the biological make-up of circulating leukaemic leucocytes (and therefore presumably also of those in the haemopoietic organs), and that these changes could be observed *in vitro*. Consequently, it is possible that, given standard conditions of culture, the mitotic activity of leukaemic cells *in vitro* may be found to be an index of the activity of the disease in the patient.

DISCUSSION.

The main purpose of the work reported in this paper was to determine whether doses of X-rays within the range of those used therapeutically could produce changes in cultures of human leukaemic leucocytes; whether such changes, if present, could be analysed quantitatively; and if that were so, whether any

EXPLANATION OF PLATE.

FIG. 5–14 are reproduced from photomicrographs of human leukaemic leucocytes (chronic myeloid leukaemia), in smears of 11-hour cultures. The cultures had been irradiated with 1000 r at 7 hours, and the figures show the development of degenerate forms from cells probably irradiated in prophase. Stain: Leishman-Giemsa. In the original films, the granules were stained reddish-blue, the cytoplasm was grey-blue, and the nuclear material dark purple or almost black. × 2000.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evidence about the mechanism of their production could be obtained from the experiments.

It has been seen that changes did occur in cultures as a result of doses of 100 r or higher, i.e. doses such as are employed in the treatment of leukaemia. These changes consisted in a progressive fall in the number of immature cells, over and above that in the control cultures, a fall which took place at an earlier stage and was of a more profound degree, as the dose was increased in size. This finding is in agreement with that of Osgood and Bracher (1939), who noted a similar drop in leucocyte counts after comparable doses of radiation. Schrek (1946*a*) failed to obtain this effect, but, as he himself pointed out, conditions in his cultures precluded the occurrence of cell division.

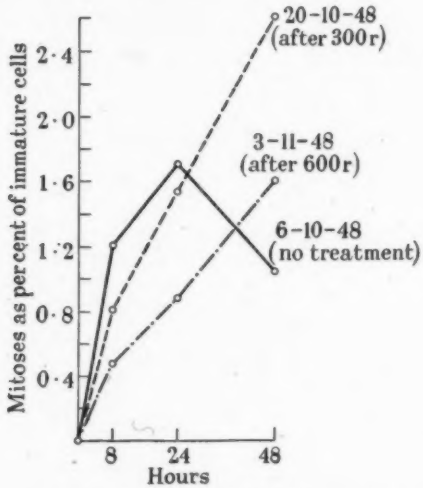

FIG. 15.—Culture of human leucocytes. Mrs. C. S—. Chronic myeloid leukaemia. Counts of mitoses in cultures made before, during and immediately after a course of X-ray treatment totalling 600 r. (The leucocyte count had dropped from 400,000 to 55,000 per c.mm.)

From an analysis of his results Osgood (1940) concluded that the fall in leucocytes after irradiation must have been due to an inhibition of mitosis. This surmise is borne out by the present results, which show clearly that mitosis was temporarily depressed by the application of 100 r of X-rays, and apparently permanently by 1000 r. It would thus appear that the observed fall in the number of immature cells was a consequence of fewer of them entering mitosis, while maturation and decay proceeded at an unchanged rate.

A temporary inhibition of mitosis is a well-recognized effect of radiation which has been demonstrated in many different test objects (Lea, 1946, Chapter 8). Tissue cultures figure prominently on the list, starting with the pioneer work of Strangeways and his collaborators; this has been fully reviewed by Spear (1935). Characteristically, cells, after a delay in mitosis which varies with the size of the dose, begin to divide again, but the further divisions may be abnormal. The action may thus be reversed, and may theoretically be due

either to the destruction of some cell constituent necessary for division, or the production of an inhibitory substance: recovery is possible in either case. Mitchell (1942, 1943) and von Euler and von Hevesy (1942) have, however, shown that therapeutic doses of X- and gamma radiations inhibit the synthesis of desoxyribonucleic acid in proliferating normal and malignant cells, and hence it appears possible that the delay in division is due to disturbances in the nucleic acid cycle, such as described by these workers.

The duration of the delay in mitosis varies not only with the dose, but also with the type of cell irradiated. Thus Spear (1935) mentions that when chick fibroblasts were irradiated with gamma rays, equivalent to a dose of about 300 r, the number of mitoses after 3 hours was 46 per cent of the controls; the effect of the same dose given to cultures of Jensen rat sarcomas was that mitoses at 9½ hours were only 0–3 per cent of the controls—a result attributed by Spear to a greater delay in mitotic recovery in the case of the neoplastic cells. In the present experiments the mitotic rate after 100 r was 0–50 per cent of controls. It must be remembered that no dividing cells were present initially, and that several hours may have been required to bring resting cells to the early stage of mitosis. Nevertheless, the delay observed is of the order noted in other similar experiments. It is considerably greater than that found by Rachmilewitz *et al.* (1945) for normal rabbit bone marrow, but these authors worked with a different culture technique.

No evidence was found that 100 r produced different results when given at different dose rates. This is in agreement with the finding of Lasnitzki (1946) that in cultures of chick fibroblasts the initial inhibition of mitosis was independent of the dose rate (9.3–103 r/min.) for doses up to 100 r. The explanation in the present experiments is probably that with both the dose rates used (62 and 622 r/min.), the time of irradiation—10 or 97 seconds—was short compared to the overall time required for mitosis (35–60 minutes).

No significant increase in the number of mitotic abnormalities was found to follow irradiation of cultures. The explanation for this is not clear; it is, however, possible that technical difficulties prevented the recognition of finer structural alterations, like chromosome breaks.

When cultures were irradiated 7 hours after their start, it was found that many cells in mitosis at the moment of irradiation completed their division. This agrees with the observations of many other workers, such as Strangeways and Oakley (1923), Canti and Donaldson (1926), Kemp and Juul (1930), and Love (1931). Furthermore, with 1000 r, a new kind of breaking-down cell appeared 3 hours after irradiation, and rapidly increased in numbers. The fact that this was not found when cells were irradiated at an earlier stage, i.e. when none or few were about to enter mitosis, suggests that these abnormal cells were in the late pre-mitotic or early mitotic stage when irradiated. Similar "degenerate" cells have been seen by Lasnitzki (1940) in tissue cultures of chick fibroblasts, and by Spear and Glücksmann (1938) in tadpoles, after irradiation. In both cases the authors derive the "degenerate" cells from cells which had just completed prophase. It appears significant that similar effects followed irradiation of cells both *in vitro* and *in vivo*—a fact which has also been noted by other authors (e.g. by Gladstone and Colwell, 1933, who irradiated whole chick embryos). It is certainly possible that the same relationship may hold true for human cells.

It must be emphasized that in none of the irradiated cultures was there any evidence for a direct destruction of resting cells, but that the only obvious effect was that on cells in mitosis. This has been generally found in tissue culture work with moderate doses, as mentioned by Spear (1935).

The fact that 100 r can produce an appreciable depression of the degree of mitosis in cultures of leukaemic cells may well be of clinical significance. Assuming, for instance, that a similar process took place in the irradiated spleen, we should expect a definite diminution in the number of new leukaemic cells produced in that organ; moreover, any further similar treatments would repeat, and possibly augment the effect. One could therefore picture the manner in which X-rays cause the enlarged spleen to involute. It is, however, more difficult to account by this means for the fall in circulating leucocytes which is such a prominent feature of the response to local radiotherapy in leukaemia, or for the involution of distant leukaemic lesions which had not been directly irradiated (for examples, see Piney and Riach, 1932). Some light may be thrown on the latter problem by the findings in Section B of the experimental part. Here it was seen that leukaemic leucocytes, taken from the peripheral blood, showed a diminished power of proliferation *in vitro* after therapeutic irradiation of the patient, and that this effect was reversed as the clinical effects of treatment began to wear off. It thus seemed as if the biological make-up of the cells had been changed by the treatment. Somewhat similar conclusions had earlier been reached by Knott and Watt (1929), who had found a diminished phagocytic power of leukaemic cells after irradiation of patients. It might well be speculated that there had been a change in the nucleic acid contents of these leucocytes, for the close connection between nucleic acid metabolism and cell division is by now well established (Caspersson and Schultz, 1939, 1940; Caspersson and Santesson, 1942; Mitchell, 1942, 1943; Davidson and Waymouth, 1944; Stowell, 1946). If, following therapeutic irradiation, leucocytes *in vivo* showed the same diminished proliferative activity, this might lead to a fall in the leucocyte count, and thus account for part of the observed clinical effect, although the precise mechanism would still require explanation. Discussion of this point is, however, beyond the scope of this article.

SUMMARY.

The paper gives the results of experiments in which leucocytes from patients with chronic myeloid leukaemia were exposed to X-rays in tissue cultures made by a previously published technique. It was found that mitosis was temporarily inhibited by a dose of 100 r, and apparently permanently by 1000 r. As a result the number of immature leucocytes declined in cultures. No mitotic abnormalities were found in irradiated cultures other than those occurring also in controls, unless special arrangements were made. In the latter case a large number of abnormal, degenerating cells appeared, which were probably derived from breaking-down prophases.

There was no sign that X-rays acted by direct destruction of resting cells.

Leukaemic leucocytes taken and cultured after therapeutic irradiation of patients showed a diminished proliferative activity *in vitro*.

The author wishes to tender his sincere thanks to Professor J. S. Mitchell, for his constant help, advice and constructive criticism during the experiments

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REFERENCES.

CANTI, R. G., AND DONALDSON, M.—(1926) *Proc. Roy. Soc.*, B., **100**, 413.
CASPERSSON, T., AND SANTESSON, L.—(1942) *Acta Radiol. Stockh.*, Suppl. 46.
Idem, AND SCHULTZ, J.—(1939) *Nature*, **143**, 602.—(1940) *Proc. nat. Acad. Sci.*, **26**, 507.
DAVIDSON, J. N., AND WAYMOUTH, C.—(1944) *Biochem. J.*, **38**, 379.
VON EULER, H., AND VON HEVESY, G.—(1942) *K. Danske, Vider Skal. Biol. Med.* **17**, Nov. 8.
GLADSTONE, R. J., AND COLWELL, H. A.—(1933) *J. Anat. Lond.*, **68**, 85.
GREGORI, A.—(1939) *Strahlentherapie*, **65**, 163.
GUNZ, F. W.—(1948*a*) *Brit. J. Cancer*, **2**, 29.—(1948*b*) *Ibid.*, **2**, 41.
JANSSON, G.—(1927) *Acta Radiol.*, **8**, 427.
JOLLY, J., AND LACASSAGNE, A.—(1923) *C.R. Soc. Biol. Paris*, **89**, 379.
KEMP, T., AND JUUL, J. (1930) *Acta Path.*, **7**, 279.
KNOTT, F. A., AND WATT, W. L.—(1929) *Brit. med. J.*, **1**, 542.
LACASSAGNE, A., AND GRICOUROFF, G.—(1927) *J. Radiol. Électrol.*, **11**, 573.
LASNITZKI, I.—(1940) *Brit. J. Radiol.*, **13**, 279.—(1946) *Ibid.*, **19**, 250.
LEA, D. E.—(1946) 'Actions of Radiations on Living Cells.' London: Cambridge University Press.
LINSER, P., AND HELBER, E.—(1905) *Dtsch. Arch. klin. Med.*, **83**, 479.
LOVE, W. H.—(1931) *Arch. exp. Zellforsch.*, **11**, 435.
MELDOLESI, G., AND GIUSTI, M.—(1934) *Pathologica*, **26**, 235.
MITCHELL, J. S.—(1942) *Brit. J. exp. Path.*, **23**, 285, 296, 309.—(1943) *Brit. J. Radiol.*, **16**, 339.
NEUMANN, A.—(1924) *Strahlentherapie*, **18**, 74.
ONO, J.—(1929) *Trans. Jap. path. Soc.*, **19**, 172.
OSGOOD, E. E.—(1940) *Proc. Soc. exp. Biol. N.Y.*, **45**, 131.—(1942) *Amer. J. Roentgenol.*, **48**, 214.
Idem, AEBERSOLD, P. C., ERF, L. A., AND PACKHAM, E. A.—(1942) *Amer. J. med. Sci.*, **204**, 372.
Idem AND BRACHER, G. J.—(1939) *Ann. int. Med.*, **13**, 563.
PINEY, A., AND RIACH, J. S.—(1932) *Brit. J. Radiol.*, **5**, 393.
RACHMILEWITZ, M., ROSIN, A., GOLDHABER, G., AND DOLJANSKI, L.—(1945) *Proc. Soc. exp. Biol. N.Y.*, **59**, 129.—(1947) *Amer. J. Roentgenol.*, **58**, 464.
SCHREK, R.—(1946*a*) *Radiology*, **46**, 395.—(1946*b*) *J. cell. comp. Physiol.*, **28**, 277.
SPEAR, F. G.—(1935) *Brit. J. Radiol.*, **8**, 68, 280.
Idem AND GLÜCKSMANN, A.—(1938) *Ibid.*, **11**, 533.
STENSTROM, W., AND KING, J. T.—(1934) *Proc. Soc. exp. Biol. N.Y.*, **31**, 909.
STOWELL, R. E.—(1946) *Cancer Res.*, **6**, 426.
STRANGEWAYS, T. S. P., AND OAKLEY, H. E. H.—(1923) *Proc. Roy. Soc.*. B, **95**, 373.

MAMMARY TUMOURS IN HYBRID MICE: GROWTH AND PROGRESSION OF SPONTANEOUS TUMOURS.

L. FOULDS.

From the Laboratories of the Imperial Cancer Research Fund, London, N.W.7.

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PREVIOUS observations (Foulds, 1947, 1949*b*) showed that the growth of some transplanted mammary tumours in hybrid mice depended on a hormonal stimulus which operated in normal female mice but not in normal males; artificially-administered oestrogen supplied the necessary stimulus for growth in males. Some of the tumours were independent of the hormonal stimulus at the first transplantation; others lost their dependence after several transfers. Dependence on hormones seemed to characterize one stage in the life-history of mammary tumours in hybrid mice. The present paper deals with spontaneous mammary tumours growing in their original hosts; it records cycles of growth and regression of tumours in successive pregnancies, and a progression of the tumours towards independence of hormonal stimuli.

MATERIAL AND METHODS

The investigation comprised repeated measurements of tumours in 275 mice which carried, before death, a total of 655 mammary growths. The majority of the mice were BR hybrids, mostly of the F_3 and F_4 generations, and made up of 71 mice of strain BR4 (pink label), 90 of strain BR4 (blue label), and 69 of strain BR6. The remaining mice were 28 RB F_1 hybrids and 17 BA F_1 hybrids. A preceding paper (Foulds, 1949*a*) describes the source of these hybrids and the incidence of tumours in them.

The mice were subjected to forced breeding and most of them had pregnancies in rapid succession throughout the period of observation, even when bearing multiple large tumours. Some intermissions of breeding were attributable to temporary absence of males or to infertility of the males.

The mice were inspected twice weekly. Tumours were recognized usually when about 0·5 cm. in diameter, and weekly thereafter two main diameters were measured with calipers. Growth charts were made by plotting the sum of the two diameters against time. Post-mortem measurements usually agreed satisfactorily with those made during life; measurements on regressing tumours were the most difficult and subject to the largest errors. Frequent, often daily, measurements were made on selected mice.

Litters were recorded each morning except on Sundays and public holidays. The time of parturition as shown on the charts was usually later than the actual time by unknown periods of up to 24 hours on weekdays and 48 hours at week-ends. A more accurate timing was not attempted systematically, but was sometimes achieved, by chance, when mice were examined during parturition.

Some litters, especially those promptly devoured by their mothers, were not recorded, and others, probably, were attributed to the wrong mothers. These errors were minimized in selected groups of mice examined frequently during pregnancy. Interruption of breeding was ensured in other groups by segregation of the females.

Some mice died, but most were killed when they appeared ill, when ulceration was present or threatening, or when large tumours seemed unlikely to yield additional information. The mice were examined post-mortem except for a few that were eaten; the tumours were measured and pieces of them fixed for histological examination.

In the charts the first tumour, or largest of contemporaneous tumours, is represented by a continuous line joining crosses which indicate the actual measurements; the second tumour is represented by a broken line and circles, and the third by a dotted line and triangles. To avoid overcrowding of the diagrams, tumours beyond the third are omitted from the reproductions. Vertical broken lines, headed "L," show the recorded litter-dates. The charts are reduced to approximately the same extent for reproduction except for Fig. 19, 21, 30, 40 covering especially long periods of observation, which are reduced to a greater degree. Scales of time and size are shown on each chart. The legends give the situations of the tumours which are numbered (i) continuous line, (ii) broken line and (iii) dotted line.

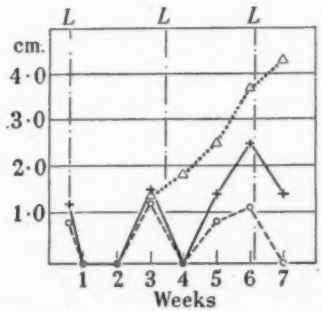

FIG. 1.—BR6, F_4, No. 175. (i) Right axilla; (ii) left groin; (iii) right neck.

RESULTS.

General Features of Growth.

Some tumours grew steadily from their first appearance until the death of their hosts; as described by Haddow (1938), their course was represented on the charts made by plotting the sum of 2 diameters against time by an approximately straight line (Fig. 1, dotted line). Other tumours, constituting a majority, were conspicuously different. Discovered, as a rule, during pregnancy, they disappeared after parturition, recurred during the next pregnancy, grew to a peak at or near the time of parturition and thereafter regressed again, partially or completely. Some tumours repeated the cycles of growth and regression in almost identical form in several successive pregnancies (Fig. 1, broken line).

Others grew to a higher peak in each succeeding pregnancy, the charts showing ascending curves with peaks near the times of parturition and intervening troughs (Fig. 1, continuous line). Tumours sometimes grew in that way throughout their course. Many tumours, however, changed their course conspicuously, and apparently abruptly, during the period of observation; after several cycles of growth and regression with equal peaks they continued in cycles with steadily-ascending peaks (Fig. 2, continuous line) or, after pronounced waves of growth and regression in several pregnancies, they grew progressively without acceleration at succeeding pregnancies and without regression between pregnancies, their new course being represented on the charts by a straight line (Fig. 3, continuous line).

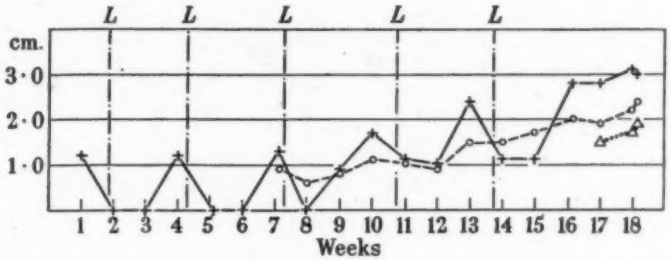

FIG. 2.—BR4 (blue label), F_4, No. 145. (i) Right groin; (ii) right vulva; (iii) left neck.

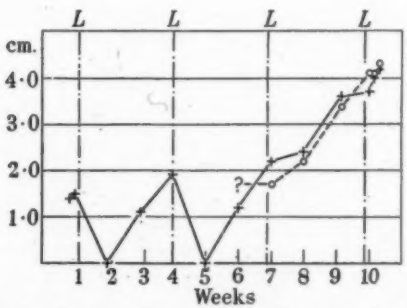

FIG. 3.—BR6, F_4, No. 178. (i) Left axilla; (ii) right axilla.

In most of the mice from 1 to 6 new tumours appeared during the course of the first. The second and subsequent tumours were as varied in type as those which appeared first. The several tumours in the same mouse did not conform to a single type of growth nor to a regular sequence of types. Fig. 1 shows three different types of behaviour at the same time in one mouse.

At least three major factors contributed to the wide diversity of behaviour amongst spontaneous tumours, and even amongst multiple tumours in the same mouse: first, the regulation of tumour growth by the environmental, and presumably hormonal, changes occurring during pregnancy and the puerperium; second, the specific reactivities of particular tumours to the environmental

changes; and third the qualitative changes occurring in tumours during their manifest clinical course as evidenced in particular by altered responsiveness to reproduction. In the remainder of this paper the terms "responsive" and "unresponsive" are used in a special and restricted sense to describe tumours which, respectively, do and do not vary their growth in response to the physiological changes occurring in their hosts during pregnancy and the puerperium. The term "progression" is used, also in a special sense, to denote *qualitative* change in tumours as distinguished from mere advancement in size, and as evidenced in particular, but not exclusively, by altered responsiveness to pregnancy and parturition.

Subsequent paragraphs detail the analysis of the phenomena here outlined. For convenience of description the tumours are classified according to a few main types of behaviour, but transitional and indeterminate types are encountered and preclude a precise statement of the relative frequency of types. The primary division is between "responsive" and "unresponsive" tumours. On a rough estimate, the ratio of responsive to unresponsive types is about 3:1 in BR and RB hybrids, and close to unity (16:13) in BA F_1 hybrids.

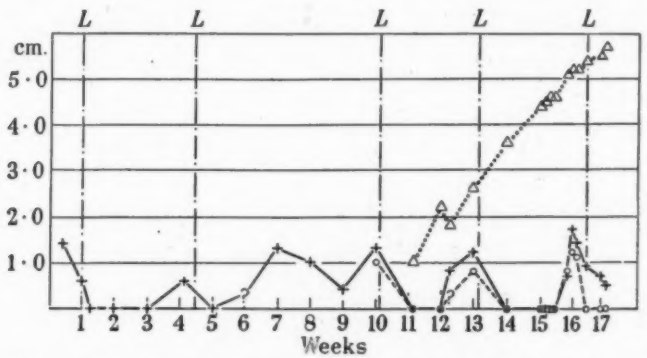

FIG. 4.—BR6, F_3, No. 2. (i) Right axilla; (ii) right groin; (iii) right shoulder. (Two late tumours omitted.)

Unresponsive Tumours.

The unresponsive tumours comprised the *primarily unresponsive tumours* which were unresponsive when first recognized (Fig. 1, dotted line; Fig. 3, broken line), and the *secondarily unresponsive tumours* which, earlier, had been responsive (Fig. 3, continuous line). The growth of an unresponsive tumour was represented by an approximately straight line undeflected during pregnancy or the puerperium. Growth was often rapid or of moderate rate (Fig. 4, dotted line), but was sometimes extremely slow, the change in size from week to week being scarcely perceptible (Fig. 2, broken line).

Closely-spaced measurements showed complete absence of response to pregnancy and parturition (Fig. 4). Less frequent measurements did not exclude minor responses, but the contrast between responsive and unresponsive tumours in the same mouse was usually unmistakable, as shown in many of the charts (Fig. 1, 2, 4, 10). Some charts, however, were equivocal, showing minor

fluctuations not certainly outside the limits of error in measurement, but possibly indicating tumours of transitional type linking the frankly responsive and unresponsive groups.

With rare exceptions unresponsive tumours in the same mouse, whether primary or secondary, ran parallel courses (Fig. 5, 6).

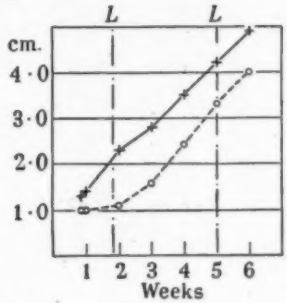

FIG. 5.—BR6, F_3, No. 34. (i) Left groin; (ii) right groin.

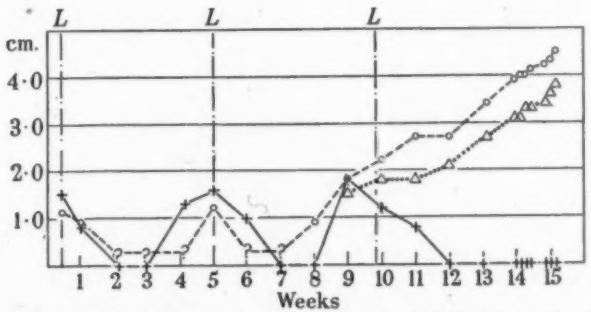

FIG. 6.—BR4 (pink label), F_4, No. 85. (i) Left neck; (ii) left groin; (iii) right groin.

Responsive Tumours.

The responsive tumours were characterized by growth during pregnancy to a peak close to the time of parturition and regression to a greater or less degree during the puerperium. They were separable for purposes of description into three principal groups designated Responsive Types, I, II and III respectively.

Responsive Type I.

The responsive Type I tumours repeated the cycle of growth and regression in almost identical form in successive pregnancies. The peaks of growth during pregnancies were at about the same level, and the intervening regressions were usually, but not invariably, complete (Fig. 4, continuous line). The tumour represented by the broken line in Fig. 7 completed 5 cycles with similar peaks and, at the end, was no larger than when it was first seen 18 weeks previously.

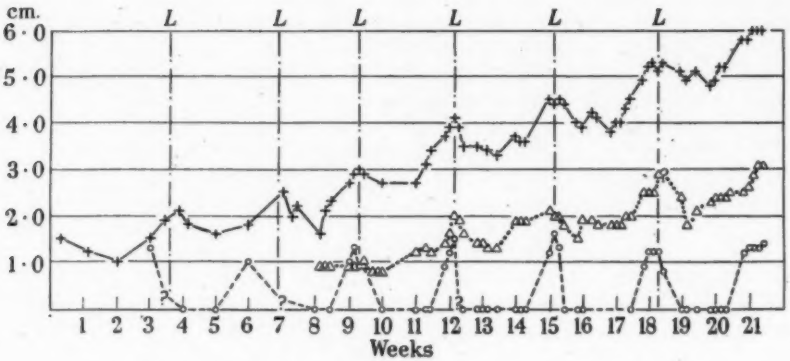

FIG. 7.—RB, F_1, No. 61. (i) Right groin; (ii) right vulva; (iii) left axilla.

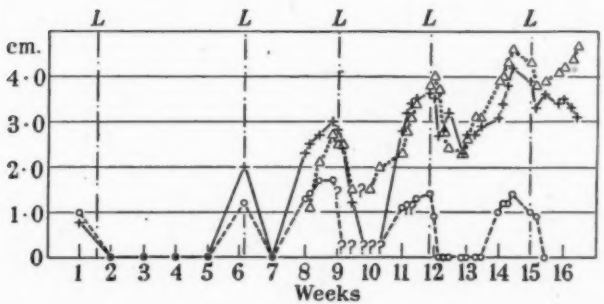

FIG. 8.—BR4 (pink label), F_2, No. 211. (i) Left axilla; (ii) left groin; (iii) right vulva.

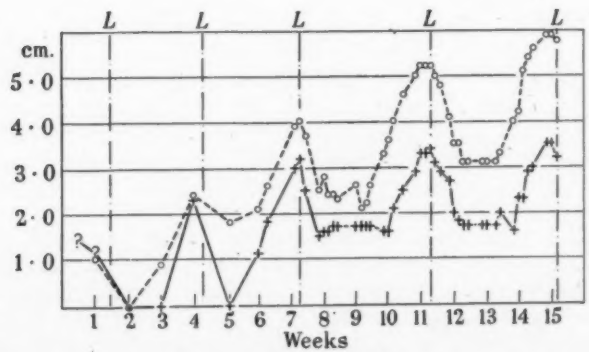

FIG. 9.—BR4 (pink label), F_2, No. 73. (i) Left axilla; (ii) right groin.

The smallest tumour shown in Fig. 8 (broken line) behaved similarly. The peak size, however, was often considerable, as shown in some of the other charts representing Type I tumours (Fig. 20). Some tumours maintained their course unchanged through several months of observation, whereas others after two or three cycles changed their behaviour, as described in a later section.

The growth of Responsive Type I tumours was strictly conditional and dependent on pregnancy. If breeding stopped the tumours disappeared and, as a rule, did not recur until breeding was resumed. The responsiveness to pregnancy and parturition remained constant, and there was no net advance in size or in aggressiveness.

Responsive Type II.

The responsive Type II tumours grew to a higher peak at each successive pregnancy but they remained responsive throughout. The range of response at each pregnancy was nearly constant for the same tumour, but varied considerably from one tumour to another. Fig. 7 (continuous line) illustrates a tumour

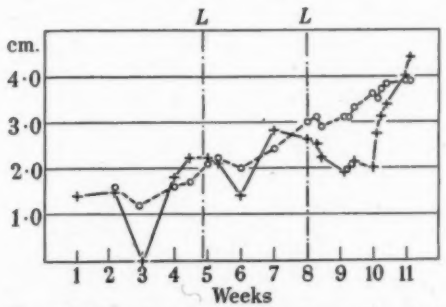

FIG. 10.—RB, F_1, No. 51. (i) Right vulva (? recurrence of tumour excised 7 weeks previously; earlier tumour not shown); (ii) right axilla.

with a small range of growth and regression at each pregnancy, and a slow but steady increase in average size. Another tumour shown in the same diagram (dotted line) appeared 8 weeks after the first and then followed an almost parallel course. The range of pregnancy response and the net growth in each cycle were sometimes much greater, as shown in Fig. 8 and 9. Other examples of Type II tumours are shown in Fig. 1, 2 and 30. Many tumours that were examined only at weekly intervals evidently belonged to this group, although the details of individual cycles were not shown (Fig. 10, continuous line).

The distinctive features of the Type II tumours were their persistent responsiveness and steadily-mounting growth-curves. The peaks of growth, or the averages of the maximum and minimum sizes during successive pregnancies, fell close to a straight line. Thus, the course of a tumour was represented by a curve which could be resolved into a straight line with superimposed waves; the straight line indicated the rate of net growth or, for want of a better term, the *intrinsic growth rate*, and the waves indicated the response to pregnancy and parturition. The intrinsic growth rate as measured by the slope of the straight line, and the responsiveness, as measured by the amplitude of the waves

remained constant in Type II tumours; the tumour illustrated in Fig. 7 (continuous line) did not notably change its intrinsic growth rate or its responsiveness during the observation period of 21 weeks. Type II tumours in the same mouse had similar intrinsic growth rates (Fig. 7, 8). The intrinsic growth rate and the degree of response, however, varied within wide limits and independently of each other from one mouse to another. Similar intrinsic growth rates were linked with different degrees of responsiveness and vice versa. Responsive and unresponsive tumours in the same mouse sometimes, as an exception to the general rule, had similar intrinsic growth rates as illustrated in Fig. 10; the wavy curve of the responsive tumour repeatedly crossed the straight line of the unresponsive tumour, but the net growth, after two months, was about the same.

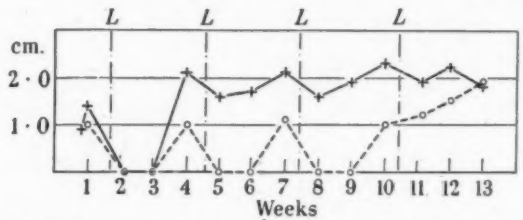

FIG. 11.—BR4 (blue label), F_4, No. 179. (i) Right axilla; (ii) right groin.

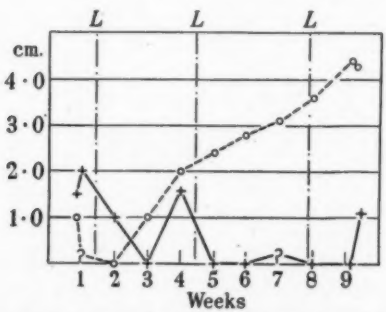

FIG. 12.—BR6, F_4, No. 136. (i) Right neck; (ii) left neck.

Responsive Type III.

Responsive Type III tumours maintained a steady average size for considerable periods of time and responded weakly to pregnancy. Their course was represented by a horizontal straight line with superimposed shallow waves (Fig. 11, continuous line). They were rare, in contrast with Types I and II, which were common. Their differentiation from Type I tumours was questionable, but they seemed to correspond with relatively frequent tumours in non-breeding mice whose course was represented by a horizontal straight line, and possibly differed essentially from Type I tumours in the ability to *persist* without the stimulus of pregnancy. The discrepancy in the frequencies in breeding and non-breeding mice might be attributed to the transience of Type III behaviour in breeding mice.

The growth of almost all the tumours encountered in the investigation could be analysed into various sequences of the unresponsive and responsive types already described. The great diversity of behaviour was attributable to two circumstances: first, the intrinsic growth rates and the responsiveness varied within wide limits and independently of each other as shown in the present section and, second, progression from one type to another occurred variously during the clinically manifest course of the tumour. The section which follows deals with progression.

Progression.

Progression in responsiveness.

After courses of varied duration, tumours of responsive Types I and II changed into secondarily unresponsive tumours (Fig. 3, continuous line; Fig. 6, 11, 12, broken lines). The tumours grew according to expectation during a pregnancy but did not regress after parturition; instead, growth continued steadily and progressively. In the diagrams the curve rose to the usual peak

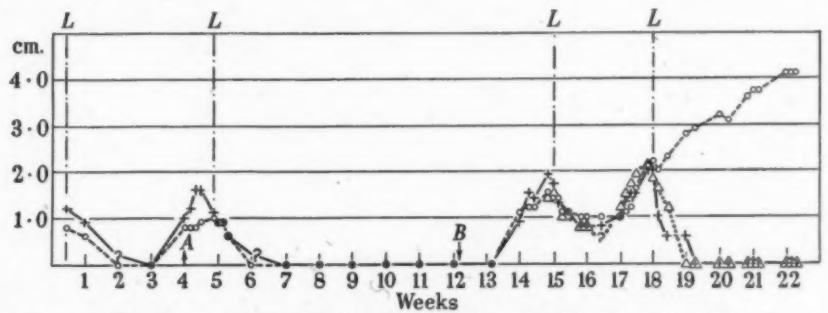

FIG. 13.—BR4 (blue label), F_4, No. 24. (i) Left groin; (ii) right groin; (iii) right vulva. Male removed at A, replaced at B.

at the end of pregnancy, and then continued as a straight line somewhat less steep, as a rule, than the ascending limb of the pregnancy wave (Fig. 11, 12). During the last pregnancy shown in Fig. 13 three tumours grew to a peak, and their curves, up to that point, so nearly overlapped that they are distinguished with difficulty in the reduced diagram. Then two tumours declined rapidly from the peak; they were undetected 9 days after parturition, and were not found again during life or post-mortem. The remaining tumour grew progressively from the peak. Evidently the decisive change was in one of three tumours and not in the environment, to which the other two tumours reacted as they had done before.

Those altered tumours that were tested by subsequent pregnancies proved unresponsive (Fig. 12); the other tumours were represented by the curves, approximating to straight lines, characteristic of unresponsive tumours. Growth was often rapid.

With rare and doubtful exceptions, abrupt progression from responsive to unresponsive type was not observed in more than one tumour in the same mouse at the same time.

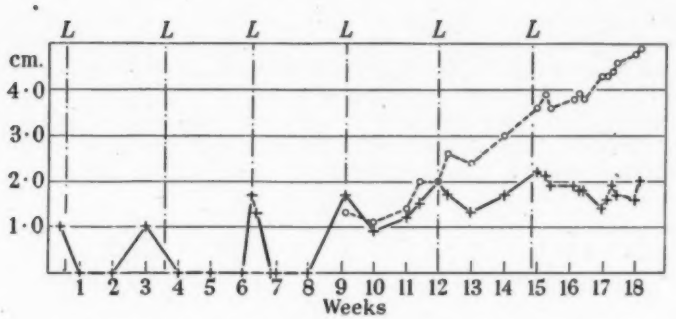

FIG. 14.—RB, F_1, No. 56. (i) Right axilla; (ii) right groin.

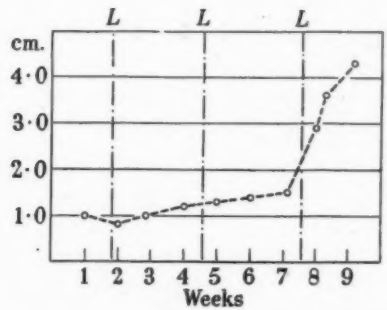

FIG. 15.—BR4 (blue label), F_4, No. 37. (ii) Left vulva (earlier tumour (i) not shown; excised 13 weeks previously; no recurrence).

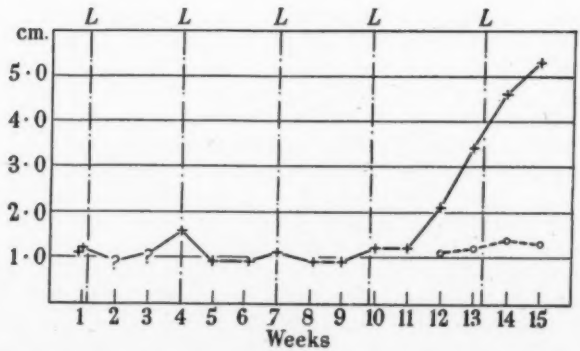

FIG. 16.—BR4 (pink label), F_4, No. 164. (i) Right groin; (ii) left axilla.

Rarely, after one or more complete regressions, a responsive tumour recurred as usual during pregnancy and continued its course as a weakly responsive Type III tumour. The change was apparently abrupt, and involved a diminution of the dependence on pregnancy without complete loss of responsiveness (Fig. 14, continuous line). A more gradual loss of responsiveness was sometimes recorded, and resulted in a responsive Type III tumour or a doubtfully unresponsive tumour.

Progression in intrinsic growth rate.

The rate of growth of two slow-growing unresponsive tumours changed abruptly (Fig. 15, 16). The acceleration was not attributable to hormonal or other environmental factors for, as shown in Fig. 16, a newly manifested tumour continued the almost horizontal straight line which represented the earlier course of the first tumour. The tumour itself had changed.

The recognition of a similar change in responsive tumours was necessarily more difficult because the pregnancy waves obscured the intrinsic growth rate. Probably an acceleration of growth often accompanied the loss of responsiveness

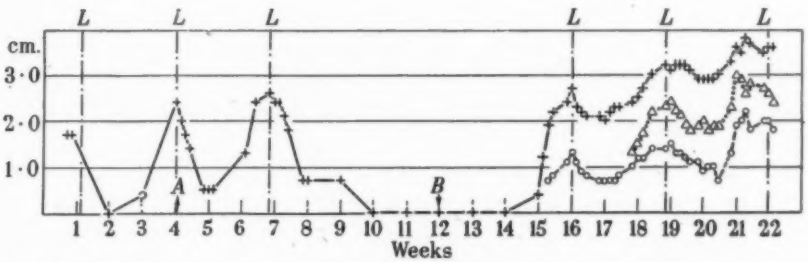

FIG. 17.—BR4 (pink label), F_4, No. 55. (i) Right vulva; (ii) right vulva (separate from (i); (iii) left axilla. (One tumour omitted.) Male removed at A, replaced at B.

described in the preceding section, but the secondarily unresponsive tumours were not necessarily rapid growing. Responsiveness and intrinsic growth rate were independent variables. The difference between the tumours described as responsive Types I and II respectively was more probably in the intrinsic growth rate than in the responsiveness. Some Type I tumours after one or more complete regressions changed into Type II tumours (Fig. 2). The change seemed abrupt and did not abolish the responsiveness. It was manifested at an early or late pregnancy in continuously breeding mice, or at the first or later pregnancy after an interruption of breeding (Fig. 17, 18).

Interrupted breeding.

When breeding was interrupted most of the tumours that regressed promptly and completely after the last pregnancy did not recur until breeding was resumed, whereupon, with rare exceptions, they reappeared during the first pregnancy. Some tumours then grew almost exactly as they had done before breeding was stopped; no progression had occurred in the interim (Fig. 13, 19, 20). Others, however, recurred at the first pregnancy as unresponsive or dubiously responsive tumours (Fig. 21, 25). Many tumours between the extremes recurred with altered

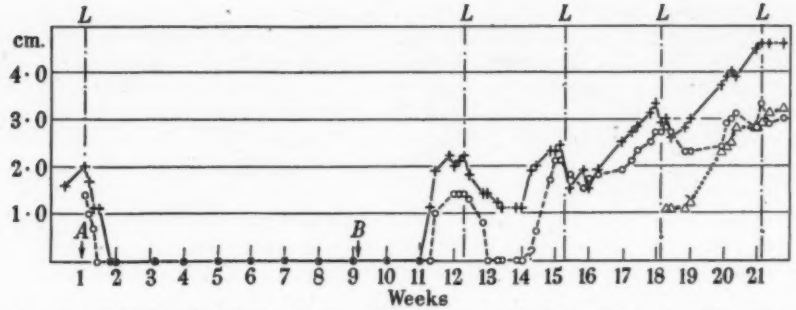

FIG. 18.—BR4 (blue label), F_4, No. 47. (i) Left vulva; (ii) right axilla; (iii) right groin. (Two tumours omitted.) Male removed at A, replaced at B.

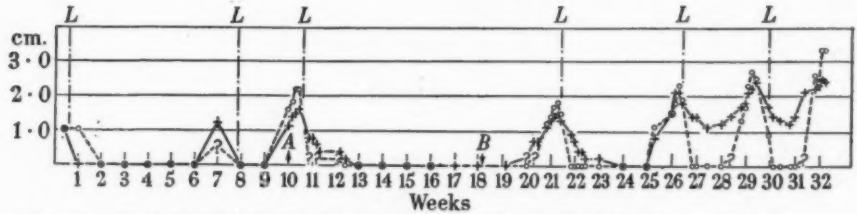

FIG. 19.—BR4 (blue label), F_3, No. 12. (i) Left axilla; (ii) right vulva. (One tumour omitted.) Male removed at A, replaced at B.

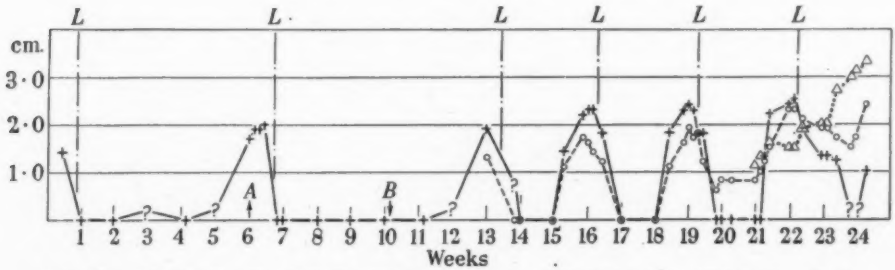

FIG. 20.—BR6, F_4, No. 49. (i) Left vulva; (ii) left groin; (iii) right vulva. Male removed at A, replaced at B.

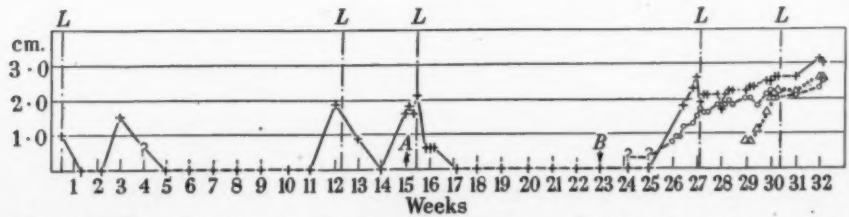

FIG. 21.—BR4 (pink label), F_4, No. 215. (i) Left vulva; (ii) left groin; (iii) left vulva (separate from (i)). Male removed at A, replaced at B.

behaviour, a responsive Type I tumour reappearing, for example, as a responsive Type II tumour (Fig. 17). Some tumours, which recurred without evidence of progression, changed at a subsequent pregnancy. The tumour illustrated in Fig. 18 (continuous line) apparently increased its intrinsic growth rate after the second pregnancy following the interruption of breeding.

A minority of tumours recurred during the intermission of breeding, almost immediately or at varied intervals after the last pregnancy. All these recurrent tumours were of unresponsive type (Fig. 22, 23, 24, 26), and they were exactly

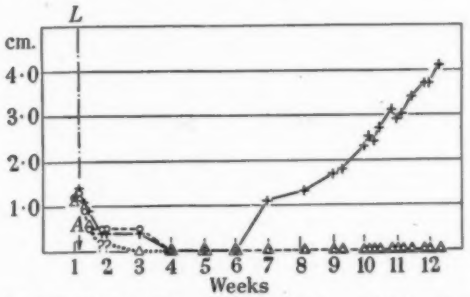

FIG. 22.—BR4 (pink label), F_4, No. 192. (i) Right axilla; (ii) left axilla; (iii) right groin. Male removed at A.

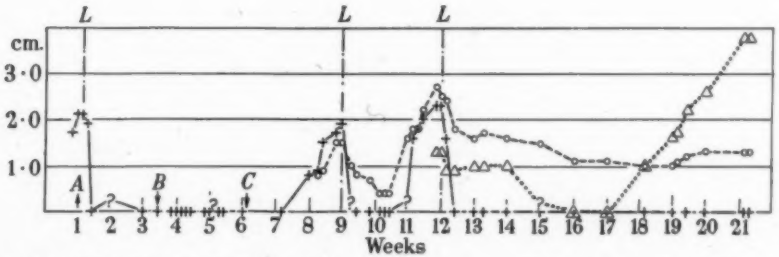

FIG. 23.—BR4 (blue label), F_4, No. 144. (i) Right vulva; (ii) left axilla; (iii) right groin. Male removed at A. Vasectomized male added at B and replaced by normal male at C.

comparable with the previously-mentioned tumours which recurred in unresponsive form at the first pregnancy when breeding was resumed. When two or three tumours had regressed only one of them recurred as an unresponsive tumour, whether during the intermission of breeding or at the first pregnancy thereafter. Fig. 26 and 25 illustrate tumours which recurred during an intermission and at the succeeding pregnancy respectively. The close resemblance indicated that progression occurred independently of breeding, although the stimulus of pregnancy was often needed to initiate the recurrent growth whereby the change which had already occurred was made manifest.

Tumours that did not regress promptly or completely after the last pregnancy behaved variously during the intermission of breeding. Regression of some

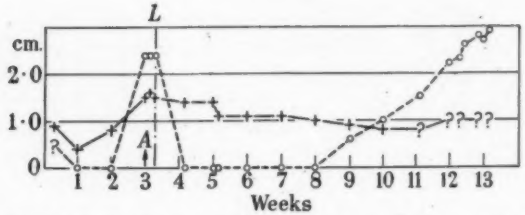

FIG. 24.—BR4 (blue label), F_4, No. 75. (i) Right vulva; (ii) left vulva. Male removed at A.

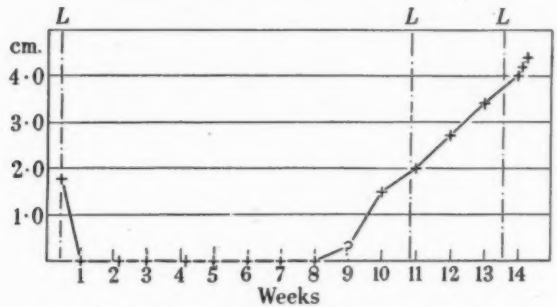

FIG. 25.—BR4 (blue label), F_3, No. 26. (i) Left vulva.

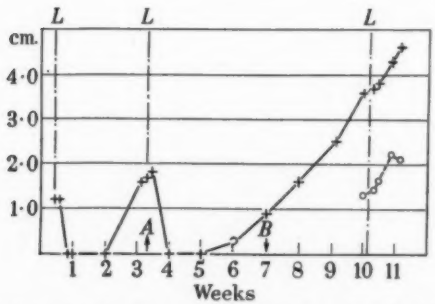

FIG. 26.—BR4 (blue label), F_4, No. 90. (i) Right vulva; (ii) left axilla. Male removed at A, and replaced at B. Nursed litter from A to B.

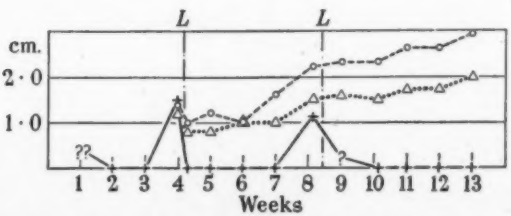

FIG. 27.—BR4 (pink label), F_4, No. 119. (i) Right axilla; (ii) left neck; (iii) left axilla. (One tumour omitted.)

tumours was slow and prolonged but eventually complete (Fig. 17, 19, 22, 23, 28). In continuously breeding mice similar regression would be incomplete when recurrent growth was started by another pregnancy; a consequent summation of the effects of successive pregnancies might account for the ascending waves of responsive Type II tumours. In two tumours shown to the right of Fig. 19, however, the one that regressed completely between pregnancies (broken line) increased its peak size somewhat more steeply than the tumour that regressed incompletely (continuous line). Increasing peak size, therefore, was not attributable, in general, to summation.

The regression of some tumours was slight and of short duration, being followed almost at once by progressive unresponsive growth. The regression was sometimes trivial and probably within the limits of error in measurements, and the tumours were then scarcely distinguishable from those already described which continued as unresponsive tumours from the peak of a pregnancy wave. Possibly the two tumours illustrated in Fig. 27 (broken and dotted lines) that grew progressively after apparent slight regression were primarily unresponsive

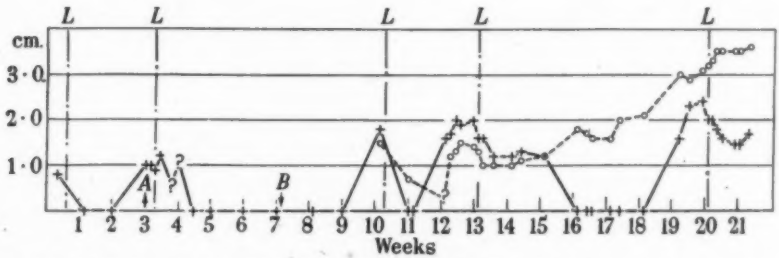

FIG. 28.—BR6, F_4, No. 74. (i) Right axilla; (ii) left vulva. Male removed at A, replaced at B.

tumours. The regression of other tumours, though brief, was more distinct (Fig. 28, broken line). In yet other tumours the regression was more prolonged, and a gradual decline from the peak size at the last pregnancy merged imperceptibly with an equally gradual resumption of growth, the course being represented by a smooth curve without an indication of abrupt change (Fig. 29, continuous line). The tumour represented by the continuous line in Fig. 29 proved unresponsive at a subsequent pregnancy. Another tumour (broken line) shown in the same figure behaved similarly, except that it apparently began to grow earlier and was less certainly unresponsive at the subsequent pregnancy.

Some tumours regressed slightly or moderately after parturition and then maintained a constant size. The course of these tumours, which possibly corresponded with the responsive Type III tumours in breeding mice, was represented by a horizontal straight line. One of the tumours began to grow again during the intermission of breeding, and continued without evident acceleration through the first pregnancy when breeding was resumed. After a brief trivial regression following parturition, it grew more rapidly during the next pregnancy and was probably unresponsive (Fig. 30, broken line). The remaining tumours persisted without growth until breeding was resumed, and then behaved variously. Fig. 31 (continuous line) and 32 (broken line) illustrate tumours which were

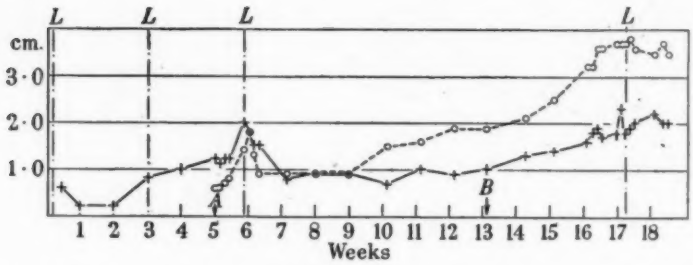

FIG. 29.—BR4 (pink label), F_4, No. 21. (i) Left vulva; (ii) right groin. Male removed at A, replaced at B.

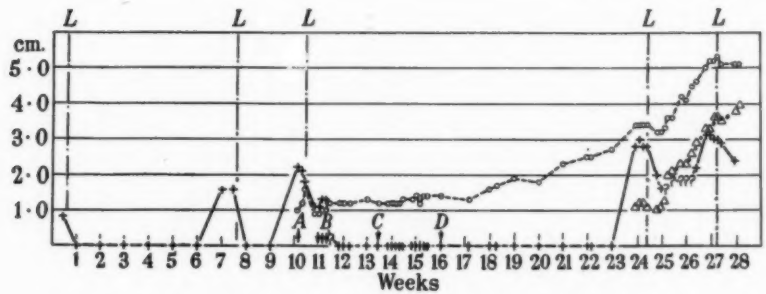

FIG. 30.—BR6, F_4, No. 50. (i) Right axilla; (ii) right vulva; (iii) left groin. Male removed at A; cutaneous applications of stilboestrol at B; vasectomized male added at C and replaced by normal male at D.

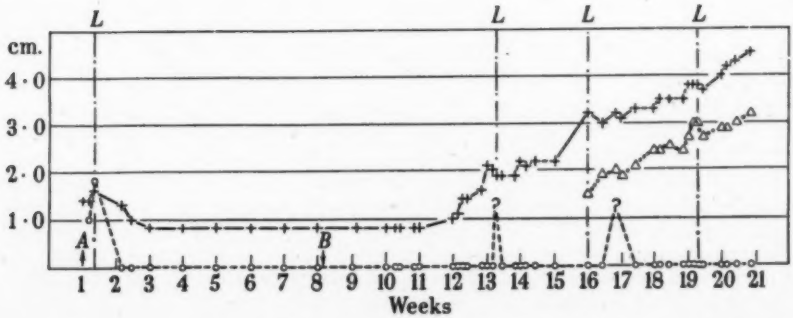

FIG. 31.—BR4 (blue label), F_4, No. 3. (i) Right groin; (ii) left axilla; (iii) left neck. Male removed at A, replaced at B.

probably unresponsive when growth resumed, the irregularities of the growth curves being attributable, most likely, to errors of measurement. The tumour shown in Fig. 33 grew as a responsive Type II tumour with unusually steep ascent. By contrast another tumour (Fig. 34) grew at the first pregnancy to a slight extent within the limits of error of measurement, but grew unresponsively through the second pregnancy and puerperium, and continued to grow unresponsively but more rapidly at the third pregnancy. The terminal course was represented by a straight line, but the intermediate course through the first and second pregnancies after the resumption of breeding was more fairly indicated by a curved line, suggesting a gradual accession of growth energy in an unresponsive (or minimally responsive) tumour.

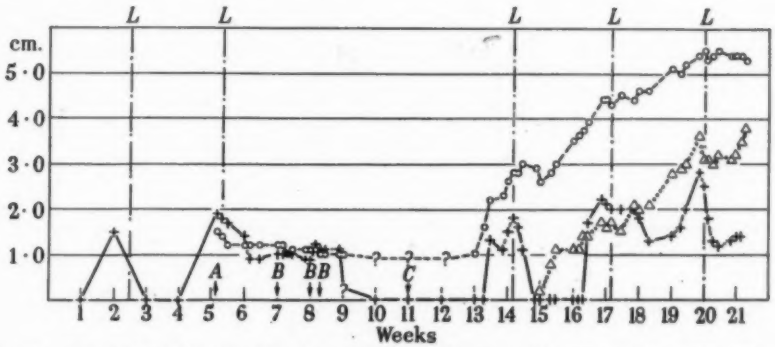

FIG. 32.—BR4 (blue label), F_3, No. 32. (i) Right axilla; (ii) left groin; (iii) right groin. (One tumour omitted.) Male removed at A; injections of theelin at B; male replaced at C.

Multiple Tumours.

Most of the mice that did not die prematurely of intercurrent infection or accident developed multiple tumours, up to 7 in number and averaging 2·3 per mouse. Sometimes 2, or less often 3, tumours appeared simultaneously or within a few days of each other (Fig. 5, 6, 22). More commonly the first tumour was solitary and further tumours developed consecutively. So far as could be seen the spacing of successive tumours and the sequence of types were irregular. The ratio of unresponsive to responsive tumours was about the same in first tumours as in second or third tumours. Tumours that remained solitary throughout the period of observation were sometimes unresponsive, but more frequently responsive. In the majority of the mice where two tumours appeared at about the same time both tumours were responsive; in the minority both were unresponsive, or one was responsive and the other unresponsive. As a rule new tumours were registered first during pregnancy.

Different types of tumours developed independently of each other in the same mouse, and progression from one type to another occurred independently in the several tumours. Some mice had, at the same time, 3 tumours of three contrasted types (Fig. 1). Progression occurred without apparent relation to the size or duration of a tumour, and indiscriminately in first, second, or later tumours in

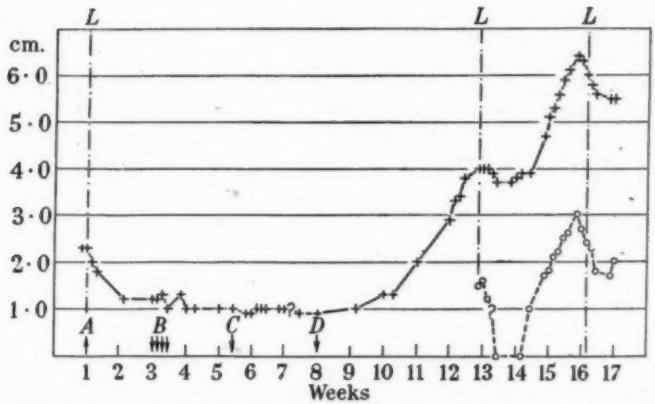

FIG. 33.—BR6, F_4, No. 42. (i) Left vulva; (ii) right vulva. Male removed at A; cutaneous applications of stilboestrol at B; vasectomized male added at C and replaced by normal male at D.

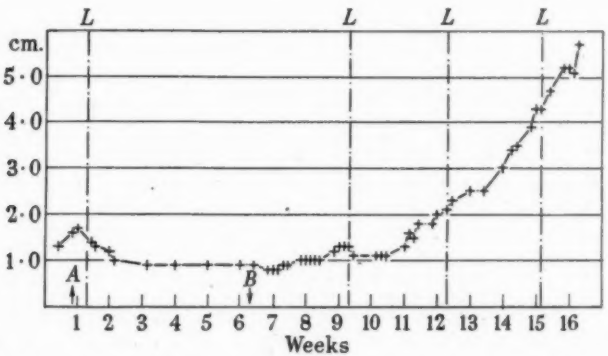

FIG. 34.—RB, F_1, No. 63. (i) Left groin (earlier tumour omitted). Male removed at A, replaced at B.

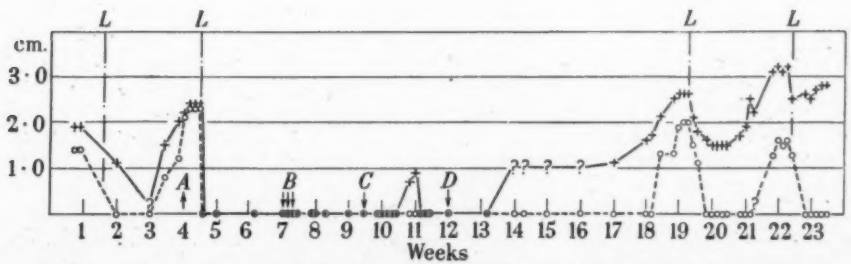

FIG. 35.—BR6, F_4, No. 11. (i) Left vulva; (ii) right vulva. Male removed at A; cutaneous applications of stilboestrol at B; vasectomized male added at C and replaced by normal male at D.

order of appearance. Abrupt progression from responsive to unresponsive type or from one responsive type to another occurred in only one tumour at a time even when two or more tumours previously had run parallel courses. Exceptions to the rule were rare and equivocal. Development of a new tumour did not noticeably alter the course of tumours already present (Fig. 1, 4). Conversely, the later of consecutive tumours were not evidently regulated by the earlier, for they were as varied in type and as liable to progression as the earlier tumours.

The preceding paragraph stresses independence and irregularity in the behaviour of multiple tumours. Observations of a different kind provide equally impressive examples of parallelism. Many diagrams illustrate parallelism of the pregnancy cycles of responsive tumours (Fig. 7, 8, 9, 13, 17). When the tumours were of similar size the growth curves overlapped so that they were difficult to distinguish in a single diagram (Fig. 13). If the sizes of the tumours and the extents of growth and regression were varied, the peak of growth, nevertheless, was reached simultaneously in all the tumours. Primarily unresponsive tumours grew in parallel (Fig. 5), and so also did primarily and secondarily unresponsive tumours in the same mouse (Fig. 3, 6.) The general rule, with few exceptions, was that *tumours of the same type in the same mouse ran parallel courses.* Tumours of different types, by contrast, did not develop in parallel; the exceptions, though conspicuous, were uncommon, and the parallelism of the intrinsic growth rates of responsive and unresponsive tumours in the same mouse (Fig. 10) was probably coincidental. In view of the extremely wide variability amongst tumours of the same type in different mice, the almost constant parallelism in the same mouse was not plausibly coincidental, but indicated either that multiple tumours were impressed at their origin with similar intrinsic growth rates, or that each was similarly regulated by environmental factors unconnected with reproduction.

The Pregnancy Effect.

Growth and regression during pregnancy and the puerperium.

The consistent feature of the growth curves of responsive tumours was a peak near the time of parturition. The ascent to the peak and the subsequent descent were sometimes remarkably steep (Fig. 23). A tumour illustrated in Fig. 14 completed one cycle of growth and regression within 6 days, and some less distinct tumours were recorded at only one or two of successive daily examinations. The peak of growth was sometimes sharp, the maximum size being maintained for not more than one day, and often rounded or flattened to a plateau lasting two or three days (Fig. 9, 24, 35). Smooth curves were obtained only when the tumours were sharp and regular in outline and conveniently situated. Subsidiary spikes on the less regular curves were probably attributable to inaccurate measurements (Fig. 7).

The most reliable and complete records indicated that the peak of growth was reached during the last day or two of gestation, and that regression began on the day of parturition. A few measurements, made by chance during or immediately after parturition, showed that regression had already started.

Exceptionally, growth seemingly continued for a day or two after parturition (Fig. 26, second pregnancy), but the increments were small, and not certainly outside the limits of error in measurement.

Regression varied widely in speed and extent. Tumours of substantial size (1·5 × 0·9 cm., Fig. 35) disappeared within 24 hours; others regressed incompletely and more gradually. As indicated by question marks in several figures the persistence, or size, of residual tumours was sometimes doubtful.

Tumours that regressed completely after parturition recurred, as a rule during the second half of the next pregnancy and often during the last week (Fig. 7, 8, broken lines). Sometimes recurrences were not detected until the penultimate or final day of gestation. Tumours that regressed incompletely behaved similarly except that growth was resumed, sometimes, during the first week of pregnancy (Fig. 8, dotted line). When another pregnancy did not follow at once a tumour, shown in Fig. 13, regressed completely within 7 days, but when another pregnancy followed without delay regression was only partial. Possibly hormonal changes during the first 2 or 3 days of pregnancy sufficed to check regression, although growth was not apparent until a few days later.

Perhaps a few of the "recurrent" tumours were, in reality, new primary growths, but so nearly as could be determined the recurrent tumours occupied the same positions as those which had disappeared and were of similar shape. After partial regression the reality of true recurrent growth was not in doubt. Some tumours were reduced to thin plaques not greatly different in superficial dimensions from the tumours at their peaks of growth; recurrence was manifested by a thickening of the whole plaque. Other tumours shrank to vague subcutaneous thickenings of doubtful, but probably considerable, extent; recurrent growth was evidenced by sharpening of the outlines. It seemed that tumours recurred by the simultaneous growth of all parts of a substantial area of previously altered mammary tissue rather than by centrifugal growth from a small residual focus. In consequence, recurrent tumours were often relatively large when first registered and the gradients of growth and regression remarkably steep.

A few tumour-bearing mice nursed and weaned their litters. Their tumours behaved during lactation like those in recently pregnant mice that did not at once become pregnant again although deprived of their young. No specific effect of lactation was seen.

Recurrence was observed, almost always, at each succeeding pregnancy, and most apparent exceptions were attributable to wide spacing of the examinations (Fig. 12). Some dubious tumours, registered once or twice on suspicion, were not seen again, but only 5 tumours, recorded on at least one occasion without query, disappeared permanently from mice that continued to breed. After interruption of breeding the tumours that had regressed recurred, with few exceptions, at the first ensuing pregnancy. Fig. 31 illustrates a tumour (broken line) that recurred doubtfully or not at all.

Some charts showed recurrences of tumours between pregnancies (Fig. 4, 21). The recurrences corresponded as a rule with expected pregnancies in continuously breeding mice, and the recurrent tumours, unlike those in segregated females, were responsive; almost certainly the recurrences were stimulated by unrecorded pregnancies. The general rule was recurrence in each successive pregnancy and no recurrence between pregnancies.

Mechanism of the pregnancy effect.

Tumour-bearing females were segregated from males, usually during pregnancy, and, after a period of observation during which tumours regressed partially or completely, they were used to test the effects of pseudo-pregnancy, gonadotropic hormone, and oestrogens. Subsequently most of the mice were mated with normal males to demonstrate the usual pregnancy response.

Eight females were mated with vasectomized males to induce pseudo-pregnancy. Brief recurrence of one tumour was recorded, but it was not comparable in degree with the recurrence at a subsequent normal pregnancy (Fig. 35). With the exception of one doubtful and transitory recurrence, no effect on the tumours was observed in the other mice (Fig. 30, 33).

Pregnancy-urine gonadotropic hormone (Antuitrin S, Parke, Davis & Co.) was injected subcutaneously into 8 mice, three doses each of 0·2 c.c. containing 20 units being given with intervals of 7 and 2 days between. The injections did not induce recurrence of any tumour that had regressed completely; tumours that regressed incompletely responded trivially and doubtfully or not at all.

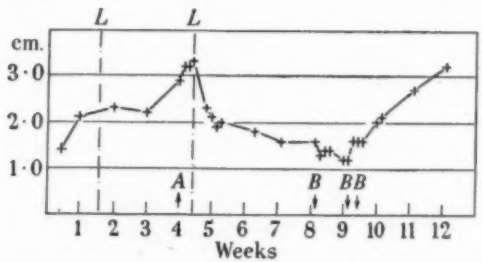

FIG. 36.—BR4 (pink label), F_3, No. 76. (i) Left groin. Male removed at A; injections of theelin at B.

Three experiments were carried out with oestrogens. In the first, each of 8 mice received 4 daily applications to the skin of 0·2 c.c. of a 0·1 per cent solution of diethylstilboestrol in acetone. The solution was old and of doubtful potency. No unequivocal effect was observed (Fig. 33, 35). In the next experiment each of six mice received three subcutaneous injections, at intervals of 7 and 2 days, of 0·05 c.c. of Theelin (Aqueous suspension, Parke, Davis & Co.), and 2 mice received single doses. Each dose contained 1000 units. No tumour that had regressed completely recurred as a result of the injections. The action on tumours that had regressed partially was uncertain. Fig. 32 suggested that the injections possibly delayed regression of one tumour, and Fig. 36 shows regression checked and succeeded by growth at the time of the injections, but as similar events occurred without injections during intermissions of breeding the interpretation was doubtful. In other mice an action on tumours was trivial or lacking.

In the third experiment cholesterol pellets containing 10 per cent of diethylstilboestrol and weighing on the average 8·2 mg. were implanted subcutaneously in 7 mice. Nine tumours previously observed in these mice had disappeared before the pellets were implanted and none recurred promptly, but eventually each mouse developed a solitary tumour. In 3 mice the late tumours recorded

54 days, 145 days and 145 days respectively after implantation of the pellets were in the same region as earlier tumours which had regressed, and were possibly, but, in view of the long delays, not probably, recurrent tumours. In the other 4 mice the late tumours were remote from the sites of the earlier tumours and were undoubtedly new primary growths. They were observed at intervals ranging from 68 days to 216 days after insertion of the pellets. It was doubtful if the oestrogen was responsible for the growth of the late tumours.

None of the experimental procedures reproduced the cycles of growth and regression which occurred in pregnancy and the puerperium. The experiments were indecisive, requiring extension with varied doses and combinations of hormones, but they discounted the possibility that growth during pregnancy was due to an oestrogenic stimulus alone.

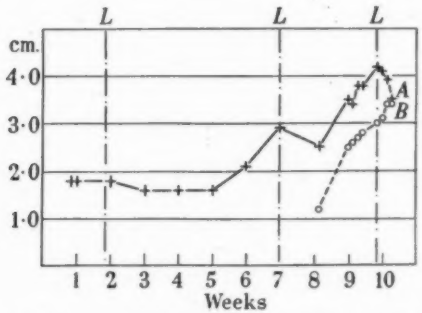

FIG. 37.—BR6, F_4, No. 90. (i) Right vulva (Tumour A); (ii) left groin (Tumour B).

Transplantation experiments.

The transplantation experiments previously recorded (Foulds, 1947, 1949*b*) showed that some tumours grew equally well in male and female hosts, whereas others grew in females or oestrogenized males but not in normal males. No other difference between tumours of the two kinds was observed, but information about the course of the primary tumours was scanty; many tumours were transplanted soon after detection, and the BR F_1 tumours together with some of the RB F_1 tumours were transplanted before systematic measurement of tumours was begun. The present section records the transplantation of tumours whose previous course was known and describes experiments with tumours removed surgically with three main objectives, namely to study autotransplantation, to compare successive primary tumours in the same host, and to compare primary tumours with the recurrent tumours which followed the operation. The experiments were few in number, the general plan of this investigation being to study the natural course of tumours with a minimum of experimental interference.

Tumours of similar size from the same mouse were used to compare the transplantabilities of responsive and unresponsive tumours. A BR6 F_4 mouse (No. 90) had a responsive tumour, A (Fig. 37, continuous line), of 9 weeks' duration; weekly measurements were insufficient to show the true peak of growth in two

pregnancies, but the more frequent measurements during the last pregnancy revealed a sharp peak and subsequent regression. Tumour B (broken line) in the same mouse grew during the last pregnancy and without interruption in the puerperium. The tumours were transplanted when almost equal in size. Tumour A grew in female hosts but not in males. In the first passage Tumour B grew equally in male and female hosts, although in the second passage the implants grew tardily in males. Tumours B and C in mouse RC12 (Fig. 38) grew respectively in females only and in males as well as females. Tumour C in mouse RC10

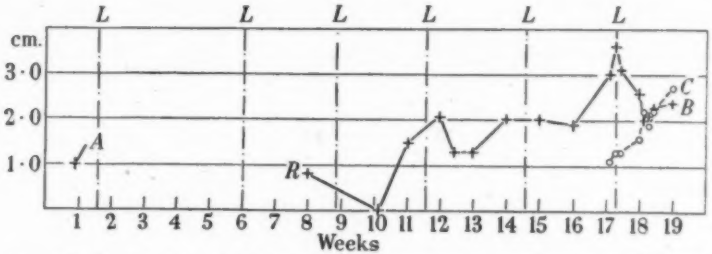

FIG. 38.—RB, F_1, No. 84. (i) Right vulva; excised at A, recurred at B (Tumour B); (ii) left axilla (Tumour C). (Two tumours omitted.)

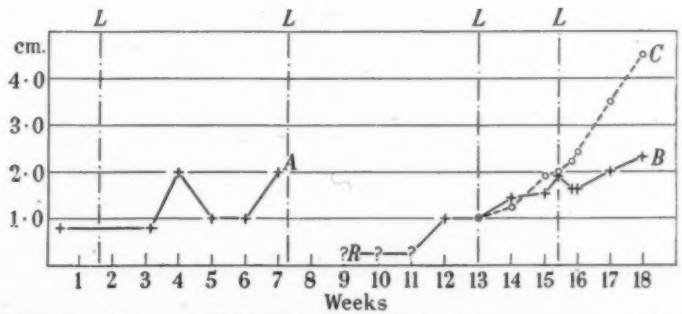

FIG. 39.—RB, F_1, No. 36. (i) Right axilla; excised at A; ? recurrence at B (Tumour B); (ii) right groin (Tumour C).

(Fig. 39, broken line) grew in males and females, but Tumour B (continuous line) grew only in females; the difference between males and females was clear-cut, although in the original host Tumour B was only weakly or doubtfully responsive. From these experiments it appeared that, in general, unresponsive tumours were transplantable into male and female hosts and responsive tumours only into female hosts, but that the correlation between type of growth in the primary tumour and behaviour after transplantation was not perfect.

Five tumours were excised and used for autotransplantation, implants being inoculated subcutaneously near the mid-line of the abdomen in order to minimize confusion with new primary tumours. Three of the transplantations were unsuccessful. The primary tumours ranged in size from 0·5 × 0·5 × 0·5 cm. to 1·0 × 0·6 × 0·4 cm., and in duration, from first detection, from 17 to 62 days;

each, so far as could be judged from the short period of observation, was "responsive." Two autotransplantations were successful. Growth of the implant from a primary tumour measuring about 0·2 cm. in diameter and removed 6 days after detection was evident after 26 days, and continued steadily until the mouse was killed 22 days later. The primary tumour from another mouse measured 0·6 × 0·4 × 0·2 cm. when excised 24 days after its recognition and was "responsive." The autotransplant was notably responsive during 5 pregnancies, but after the final pregnancy it continued to grow and, apparently, was then unresponsive (Fig. 40, broken line); ulceration of the tumour precluded observation through another pregnancy. This experiment provided evidence of progression in an autotransplant, and suggested that the delayed recurrences and delayed secondary growths observed after the excision of primary cancers from man were attributable to progression in latent tumour deposits.

Transplantation of RB F_1 tumours into BR F_1 hosts failed 3 times. The tumours ranged in size from 0·8 × 0·7 × 0·5 cm. to 1·2 × 0·9 × 0·5 cm. One of them recurred after operation, grew responsively, and, when transplanted, grew in female but not in male hosts. New primary responsive tumours developed in two of the mice; when transplanted, one of them grew in female hosts and also after pronounced delay in males, and the other grew only in females.

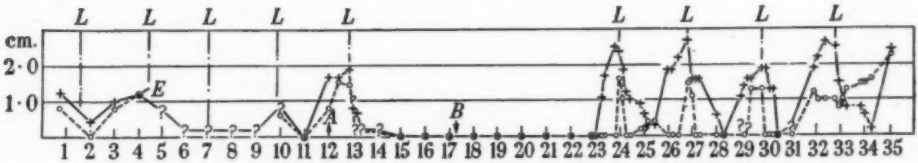

FIG. 40.—RB, F_1, No. 62. (i) Left vulva; (ii) right vulva (excised for autotransplantation at E; broken line thereafter represents the autotransplant). Male removed at A, and replaced at B.

Tumours from two RB F_1 mice (RB4 and RB10) gave evidence of progression between the transplantation of a primary tumour and subsequent transplantation of a recurrent tumour. Implants of a primary tumour excised from mouse RB4 grew only in females, whereas the recurrent tumour, transplanted 5 weeks later, grew in both males and females. Meanwhile a second primary tumour had developed at a new site. It was excised and transplanted and grew only in females, but a recurrent tumour transplanted 11 weeks later grew in females and also, though more slowly, in males. The primary tumour excised from mouse RB10 (Fig. 39, Tumour A) was transplanted successfully only into female mice, and the resulting tumours were notably influenced by pregnancy (Foulds, 1949*b*). The recurrent tumour (Fig. 39, Tumour B), transplanted 11 weeks later, likewise grew only in females, but the tumours were not responsive to pregnancy.

The experiments showed that in some mice the tumours that appeared first were less adaptable to new hosts than those that developed later, and that some recurrent tumours were more adaptable than their primary growths. The results, however, were not consistent. Successive tumours from 3 RB F_1 mice (RB6, RB7 and RB8) grew equally in male as well as female hosts; the primary tumour and two successive recurrences in mouse BR3 alike grew in females and

with slight or irregular delays in males; and although the first tumour tested from mouse RB9 grew in both males and females, a later primary tumour grew only in females.

DISCUSSION.

In the light of the present investigation it is not surprising that previous accounts of the behaviour of mammary tumours of mice during pregnancy and the puerperium are conflicting (Slye, 1920; Haddow, 1938; Baatz, 1939). In general, tumours were not followed from their first appearance through successive pregnancies, supposed effects of lactation were not controlled by observations on recently-pregnant mice deprived of their young, and contemporaneous multiple tumours were not compared. Slye combined the measurements of multiple tumours and recorded no details of individual pregnancies. As now shown, different tumours in the same mouse behave variously at the same time and the same tumour behaves variously at different times. Haddow, using in the main dealers' mice of unknown ancestry, recorded frequent measurements during and after pregnancy, and concluded that "no evidence was found to suggest that gestation itself has any influence on the rate of growth of mammary cancer in the mouse, but in approximately half of the available cases it was observed that parturition was followed by a temporary decline in tumour growth rate." Haddow's two figures illustrate, respectively, a substantial though incomplete regression after parturition and a halting of growth without regression; they match some curves of mine. Gardner (1941) described 5 hybrid (C57 black $\times$ CBA) F_1 mice which, repeatedly, had tumours attaining 1–2 cm. in 2 to 5 pregnancies; the tumours regressed after parturition, but 3 of the mice eventually died with mammary adenocarcinomas.

The responses of the tumours in the hybrid mice used in the present investigation are, possibly, exceptional in frequency and degree, but are not essentially different from those described by Haddow and by Gardner. Notable response to pregnancy and parturition is here recorded in hybrids of varied genetic constitution derived from the inbred strains C57 black, RIII and A; it is not correlated with anomalous transmission of the milk-borne mammary tumour agent.

The pregnancy effect usually becomes apparent during the second half of gestation, but probably begins during the first week and continues until the day before parturition. The abrupt regressions about the time of parturition suggest the sudden withdrawal of a stimulus which, though not yet identified, is presumably hormonal, affecting widely-distributed multiple tumours. The preliminary experiments with hormones are indecisive; a simple oestrogenic action seems unlikely, and the possible effect of gonadotropic hormones, placental hormones, and the co-operative action of oestrogenic and luteal hormones require further investigation. Lactation has no specific effect, and modifies the course of the tumours only in so far as it delays the next pregnancy.

The pregnancy effect on mammary tumours is comparable with the "promoting" action of various procedures on chemically-induced tumours of the skin of rabbits and mice. Rous and his colleagues (Rous and Kidd, 1941; MacKenzie and Rous, 1941; Friedewald and Rous, 1944*a*, *b*) studied promoting factors in rabbits, and Berenblum, who has recently adopted Rous's nomenclature, in mice (Berenblum, 1944; Berenblum and Shubik, 1947). Rous and

his colleagues describe the "promoting" action of trauma and irritants in eliciting and maintaining tumour-growth from latent tumour cells present in the skin as a result of the "initiating" action of a carcinogen. Some "conditional" tumours grow only whilst the promoting factor operates; they disappear when it is withdrawn, but recur from the irreversibly altered epithelum when the promoting factor is restored. Rous and Kidd discuss whether or not the conditional growths of rabbits' skin are "true tumours." Their argument applies equally to the conditional mammary tumours of mice. In essential agreement with Rous and Kidd, I consider that their exclusion from the group of tumours by an arbitrary definition is unwarranted, and I describe as "tumours" all the mammary growths except a few palpable nodules which regress and do not recur, and those nodules, probably similar, that do not survive autotransplantation. They are accountable to a reversible change in mammary tissue, and perhaps to the exceptional persistence in gross nodules of the properties of the hyperplastic nodules or "adenomas" which are widely accepted as precursors of mammary tumours in mice although many of them are abortive (Gardner, 1941). Browning (1948) reported that nodules less than 0·2 cm. in diameter in C_3H mice were not transplantable, and that about a third of them regressed, whereas nodules larger than 0·2 cm. in diameter never regressed. My observations differ from Browning's in revealing no correlation between behaviour and size; autotransplantation of tumours more than 0·5 cm. in diameter was unsuccessful.

The remaining mammary tumours develop from an irreversibly altered mammary tissue. The great diversity of behaviour is attributable to the interplay of factors which vary within wide limits and independently of each other. Classification into types, though convenient for description, is arbitrary, and hampered by gradations from one type to another. The primary distinction, although not absolute, is between the "responsive" tumours whose course is notably influenced by pregnancy and parturition and the "unresponsive" tumours whose growth is unaffected by reproduction.

The tumours assigned to Responsive Type I correspond with the "conditional" neoplasms of rabbits' skin. They grow during pregnancy, regress promptly after parturition, and recur, with rare exceptions, in the same place at each successive pregnancy; after several cycles of growth and regression they achieve no net increase in size or aggressiveness. The Responsive Type II tumours are less strictly "conditional"; they wax and wane at each gestation, but with a net increase of size at each cycle. The rate of net increase is steady and characteristic of the individual tumour, depending on a specific property described as the "intrinsic growth rate." The term is provisional, to be discarded when elucidation of the property suggests a better name, and is used to denote the steady growth, represented by a straight line, upon which the waves of response to pregnancy are superimposed. The intrinsic growth rate, measured by the slope of the straight line and the responsiveness as indicated by the amplitude of the pregnancy waves, are constant, often for long periods, in a particular tumour, but vary within wide limits and independently of each other from one tumour to another. The Responsive Type III tumours, of more doubtful significance, suggest a capacity for *persistence* but not for growth in the absence of promoting stimuli.

Unresponsive tumours grow progressively without acceleration during

pregnancy or retardation in the puerperium. Often they grow rapidly, but growth may be extremely slow. Responsiveness to pregnancy and intrinsic growth rate are independent variables.

Multiple tumours are of the same or of different responsive or unresponsive types, with similar or diverse intrinsic growth rates. There is no apparent regularity in the distribution of types amongst simultaneous or consecutive multiple tumours. They behave, as it seems, in complete independence of each other except that tumours of the same type in the same mouse usually follow parallel courses. This applies to unresponsive and responsive tumours alike. Tumours of the same type in the same mouse either have similar intrinsic growth rates, impressed at their origin, or they are subject to an unidentified regulation by their environment, unconnected with reproduction. "Unresponsive" is used, in this paper, in a special sense to describe tumours which do not respond to the physiological changes of pregnancy and the puerperium; it does not preclude response to other physiological or pathological stimuli.

Independent variation in responsiveness and in intrinsic growth rate accounts, in large measure, for the observed diversity of behaviour, the remaining factor of importance being progression.

I define progression as *irreversible qualitative change* in tumours. It is distinct from progressive growth or extension without qualitative changes, and from reversible alterations in structure or behaviour occasioned by extraneous stimuli. Progression is probably of frequent occurrence and fundamental importance in tumours of animals and of man, but hitherto it has not been specifically defined or analysed. The most explicit references to progressive qualitative changes in tumours are provided by Rous and Kidd (1941) and by Berenblum (1944) in their descriptions of step-like development of induced skin tumours of rabbits and mice, by Greene (1940) in his account of "progressive steps in a graded evolutionary progress" of mammary tumours in rabbits, and in a recent summary of observations on uterine tumours of rabbits (Greene and Newton, 1948) and by Browning (1948) in a study of mammary tumours in C3H mice. Greene and his colleagues and Browning detect progression mainly by transplantation experiments, and in particular by heterotransplantation into the anterior chamber of the eye, and they use the capacity for heterologous growth in the anterior chamber of the eye as a measure of independence or "autonomy" of tumours. The behaviour of a tumour in its natural host is conditioned by its independence of factors operating in that host. I am not satisfied that heterotransplantation measures the same kind of independence. Experiments with mouse tumours do not establish a consistent relationship between clinical behaviour and the results of heterotransplantation (Dyer and Kelly, 1946). Although transplantation experiments disclose progressive changes in tumours, they are not substitutes for observation of the natural course of a tumour in its original host.

A detailed review of these and other numerous and diverse indications of qualitative changes during the growth of tumours is beyond the scope of this paper; it must suffice to mention, as additional examples, the frequent qualitative changes in transplanted tumours, and, notably, in transplanted mammary fibro-adenomata in rats, in oestrogen-induced tumours of the testis in mice (Hooker and Pfeiffer, 1942), and, as I shall describe elsewhere, in vesical tumours induced in mice by 2-acetylaminofluorene. It is here proposed that these diverse phenomena, and many others, are manifestations of a fundamental characteristic

of tumours, namely the capacity for progression, as above defined, and that general principles or rules of progression, deduced from the study of mammary tumours of mice, are widely applicable.

The material for this analysis of progression consists of spontaneous tumours following their natural courses without experimental interference beyond temporary interruption of breeding. It is especially favourable on account of the abundance of multiple tumours, for the comparison of multiple tumours establishes beyond doubt that progression is a change in the individual tumour; alteration in only one of several contemporaneous growths is not attributable to change in a hypothetical "general resistance."

The varied tumours encountered in this investigation may be arranged in a series having at one extreme the wholly conditional responsive tumours, at the other extreme the rapid-growing unresponsive tumours, and intermediately all degrees and combinations of responsiveness and intrinsic growth rate. Individual tumours, however, do not in general traverse this series; they advance by abrupt steps, vaulting many possible transitional stages. At its first clinical manifestation a tumour may occupy any position in the series of types, irrespective of the time of its occurence or its position in a sequence of multiple tumours. Tumours which develop simultaneously or consecutively in the same mouse conform sometimes to the same type and sometimes to as many types as there are tumours. Progression occurs independently and unpredictably in multiple tumours in the same animal irrespective of their size or clinical duration. Progression may occur earliest in the first, second, or any subsequent member of a sequence of multiple tumours. When two or three tumours, similar in size, duration, and type of behaviour are present, progression occurs in only one of them at a time. Moreover, the progression of one tumour has no apparent effect on the course of the others. The present observations decisively controvert Browning's (1948) suggestion that a second tumour may "abrogate the autonomy" of the first.

The several recognizable characters of individual tumours undergo progression independently. Often, no doubt, responsiveness and intrinsic growth rate change simultaneously, but in some tumours progression in responsiveness occurs without progression in intrinsic growth rate and vice versa. Transplantation experiments provide further evidence of independent progression of characters, namely the responsiveness of transplanted tumours to pregnancy and the gross milky secretion in response to oestrogenic stimulation (Foulds, 1949*b*).

The development of a tumour does not always reach its end-point within the life-time of the host; progression advances further during serial transplantation (Foulds, 1949*b*). Though difficult to prove, progression from responsive to unresponsive type is probably inevitable if serial transplantation is sufficiently prolonged. Transplantation, in consequence, is of limited value for determining the stage of progression of a primary tumour, since the experimental procedure may occasion further progression.

The recurrence of previously responsive tumours as unresponsive ones indicates that progression advances in responsive tumours whose growth is suppressed. Progression is independent of growth. Moreover, pregnancy is not the sole or essential cause of progression, which is manifested sometimes during intermissions of breeding and, generally, in only one of multiple tumours exposed to the same pregnancies. Pregnancy, however, usually discloses, and possibly

accelerates progression ; it seems to exert a " trigger-action." The observations in general favour the hypothesis that the hormonal conditions of pregnancy and the puerperium determine whether or not a tumour shall grow ; they do not decide *how* it shall grow. The course of the growth elicited by pregnancy depends on specific properties which change, by progression, in latent tumour cells and in stationary tumours.

The manifestation of progression in the mammary tumours is usually abrupt, as it is in the induced skin tumours of rabbits and mice, but progression itself is not necessarily abrupt ; it is possible that cumulative gradual changes reach a threshold level at which they are first manifest. Some tumours evidence a gradual progression through a graded sequence of types. Gradual, continuous progression seems uncommon, but it is less conspicuous than the abrupt type, and more accurate and more frequent measurements might reveal it more frequently. Although the observations are few and indecisive, it seems that slow gradual progression is apt to result in sluggish, irregular, or dubiously unresponsive growth. The unequivocal rapid-growing unresponsive tumours, as a rule, are either primarily unresponsive or the result of abrupt progression from a fully responsive and, often, strictly conditional tumour. Apparently two paths of progression are available. One path leads directly to unresponsive tumour ; the tumour acquires its definitive properties early without traversing the numerous intermediate stages which are possible, and which are observed on the other path or " responsive detour." The detour leads ultimately to an unresponsive tumour, but progression along it may be slow and gradual and the end-point indeterminate, or reached only after transplantation. Abrupt progression switches many tumours from the detour to the direct path ; jumping the intermediate stages, the tumours change from the fully responsive to the definitive unresponsive type. The change of path seems the less frequent and the less decisive the further the tumour has travelled along the responsive detour. Under some circumstances at least, as shown in the experiment with stilboestrol pellets in non-breeding mice, neoplastic mammary tissue which has entered the responsive detour is less liable to develop into a progressive unresponsive tumour than mammary tissue that, at the corresponding time, is unaltered or has not progressed beyond the subthreshhold neoplastic state. It is concluded that progression may be continuous, by gradual change, or discontinuous by abrupt and often long steps, and that alternative paths of development are open.

To summarize, I propose the following Rules of Progression :

Rule I.—Independent progression of multiple tumours. Progression occurs independently in different tumours in the same animal.

Rule II.—Independent progression of characters. Progression occurs independently in different characters in the same tumour.

Rule III.—Progression is independent of growth. Progression occurs in latent tumour cells and in tumours whose growth is arrested. Two notable corollaries of Rule III are :

(i) At its first clinical manifestation a tumour may be at any stage of progression.

(ii) Progression is independent of the size or clinical duration of a tumour.

Rule IV.—Progression is *continuous or discontinuous*, by gradual change or by abrupt steps.

Rule V.—Progression follows one of *alternative paths of development.*

Rule VI.—Progression does not always reach an end-point within the lifetime of the host.

These principles, as I believe, are widely applicable to the behaviour of tumours in animals and in man. In particular they account for many vagaries which have encouraged the proposition of speculative alternatives to the orthodox pathology of tumours such as that of Delarue (1947). A few applications are here briefly indicated.

The behaviour of tumours is the resultant of multiple characters which vary, within wide limits, independently of each other and undergo independent progression. The characters include growth energy, invasiveness, capacity for dissemination, and responsiveness to environmental stimuli, of which the hormones are the most easily recognized, but not necessarily the only, examples. "Malignancy" is not a single character. The typical malignant tumour of the text-books is the result of proportionate development of all the characters proper to malignant tumours. Independent progression of characters (Rule II), however, results in disproportionate or "out-of-step" development, as for example in the "benign" tumours that metastasize and the "locally malignant" tumours that do not. The out-of-step development is especially important in carcinoma of the prostate, which, despite conspicuous growth, invasion and dissemination, is responsive to hormones. Willis (1948) criticizes the sharp division of tumours into innocent and malignant groups, and deprecates the question, "Is this tumour innocent or malignant?"; the appropriate question in his opinion is, "How innocent or malignant is this tumour?" It is even more important, I suggest, to ask, "In which characters, and to what degree in each of them, is the tumour innocent or malignant?"

Errors in the prognosis of "early" tumours are explained by the observations that progression is independent of the size or duration of a tumour, and that progression occurs without manifest growth, as in latent or stationary tumours (Rule III and corollaries). A tumour may be small in size and young in clinical duration, yet far advanced in the progression of aggressive characters. Progression without manifest growth and independently of the size or duration of tumours probably accounts, too, for the otherwise mysterious but clinically important phenomena of long-delayed recurrence and secondary growth after apparent "cure" of primary tumours. Similarly the ultimate failure of several chemotherapeutic methods after favourable early response is reasonably attributed to progression in the inhibited tumours. The analogy with the recurrence of a previously responsive mammary tumour as an unresponsive growth, during intermissions of breeding in mice, is impressive. Further elucidation of progression, especially the independent progression of characters and progression when growth is inhibited, is evidently of prime importance for the management of human cancer.

SUMMARY.

Spontaneous mammary tumours in 275 hybrid mice were measured repeatedly from their first appearance until the death of the mice. Two main types of behaviour were distinguished: "*unresponsive tumours*" grew steadily without deviation during pregnancy or the puerperium, whereas "*responsive tumours*" grew during pregnancy to a peak shortly before parturition, thereafter regressed,

and recurred at the next pregnancy. Some responsive tumours (Type I) attained similar peaks in successive pregnancies, but others (Type II) reached successively higher peaks, the rate of net growth or "*intrinsic growth rate*" remaining constant. Responsiveness and intrinsic growth rate were independent variables, and were variously combined in individual tumours. Some tumours maintained the same type of behaviour throughout their clinical course; others changed their course, often abruptly, as the result of an irreversible qualitative change in the tumours termed "*progression*." The diversity of behaviour of the spontaneous tumours was attributable, in the main, to varied combinations of responsiveness and intrinsic growth rate, and to progression in one or both of these characters. The following generalizations or *rules of progression* summarize the results of the analysis of the behaviour of tumours, especially multiple tumours, in breeding and non-breeding mice: (1) Progression occurs independently in different tumours in the same animal. (Independent progression of multiple tumours.) (2) Progression occurs independently in different characters in the same tumour. (Independent progression of characters.) (3) Progression is independent of growth, occurring in latent tumour cells and in stationary tumours. (4) Progression is continuous or discontinuous. (5) Progression follows one of alternative paths. (6) Progression of a tumour does not always reach its end-point within the lifetime of the animal.

These rules are probably widely applicable to the behaviour of varied tumours in animals and in man, and some applications are mentioned briefly. "Malignancy" is not a single character. Disproportionate or "out-of-step" development of the various characters which determine malignant behaviour (independent progression of characters) accounts for many anomalies in the behaviour of cancer in man, and progression in tumours whose growth is inhibited probably explains the ultimate failure of therapeutic measures which have a favourable immediate effect.

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REFERENCES.

BAATZ, H.—(1939) *Geburtsh. Gynäk.*, **118**, 124.
BERENBLUM, I.—(1944) *Arch. Path.*, **38**, 233.
Idem AND SHUBIK, P.—(1947) *Brit. J. Cancer*, **1**, 379.
BROWNING, H. C.—(1948) *J. nat. Cancer Inst.*, **8**, 173.
DELARUE, J.—(1947) 'La problème biologique du Cancer.' Paris (Masson et Cie).
DYER, H. M., AND KELLY, M. G.—(1946).—*J. nat. Cancer Inst.*, **7**, 177.
FOULDS, L.—(1947) *Brit. J. Cancer*, **1**, 362.—(1949*a*) *Ibid.* **3**, 230.—(1949*b*) *Ibid.* **3**, 240.
FRIEDEWALD, W. F., AND ROUS, P.—(1944*a*) *J. exp. Med.*, **80**, 101.—(1944*b*) *Ibid.*, **80**, 127.
GARDNER, W. U.—1941) *Cancer Res.*, **1**, 345.
GREENE, H. S. N.—(1940) *J. exp. Med.*, **71**, 305.
Idem AND NEWTON, B. L.—(1948) *Cancer*, **1**, 82.
HADDOW, A.—(1938) *J. Path. Bact.*, **47**, 553.
HOOKER, C. W., AND PFEIFFER, C. A.—(1942) *Cancer Res.*, **2**, 759.
MACKENZIE, I., AND ROUS, P.—(1941) *J. exp. Med.*, **73**, 391.
ROUS, P., AND KIDD, J. G.—(1941) *Ibid.*, **73**, 365.
SLYE, M.—(1920) *J. Cancer Res.*, **5**, 25.
WILLIS, R. A.—(1948) 'Pathology of Tumours.' London (Butterworth & Co.).

INDUCTION OF BREAST CANCER BY OESTROGENS AND METHYLCHOLANTHRENE IN HIGH- AND LOW-BREAST-CANCER STRAIN MICE.

L. DMOCHOWSKI AND J. W. ORR.

From the Department of Experimental Pathology and Cancer Research, Medical School, University of Leeds, and the Department of Pathology, University of Birmingham.

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THE discovery of the milk factor or mammary tumour agent (Bittner, 1936) and the demonstration that three factors, namely, the genetic factor, the hormonal factor, and the milk factor, are essential for the development of spontaneous breast cancer in certain inbred strains of mice (Bittner, 1939), was followed by investigations of the part played by the agent in the induction of breast cancer by oestrogenic hormone. Lacassagne (1939*a*), Lacassagne and Danysz (1939), Twombly (1939, 1940), and later Bittner (1940, 1941), Gardner (1941*a*), Shimkin and Andervont (1941, 1942), Dmochowski and Gye (1944), found that foster-nursing of low-breast-cancer strain mice by high-breast-cancer strain females increases the incidence of breast cancer in the low-breast-cancer strain mice treated with oestrogenic hormones. Thus male mice do not develop breast cancer, even if they are susceptible and have the mammary tumour agent, unless the hormonal or oestrogenic factor is supplied, and only few or no breast tumours are induced in low-breast-cancer strain mice, i.e. in the absence of milk factor, following treatment with oestrogenic hormones. It is probable that a quantitative deficiency of any one factor can be overcome by a relative quantitative increase in the other two factors (Shimkin, 1945).

The accelerating influence of carcinogenic hydrocarbons on the appearance of breast cancer in mice of unknown genetic constitution was first observed by Maisin and Coolen (1936) and Perry and Ginzton (1937), while Dobrovolskaïa-Zavadskaïa and Adamova (1938, 1939), were unable to observe a similar effect on inbred mice employed by them. Experiments carried out by Mider and Morton (1939), Bonser and Orr (1939), Strong and Smith (1939), Bonser (1940), Strong and Williams (1941), Engelbreth-Holm (1941), Engelbreth-Holm and Lefevre (1941), Orr (1943), Kirschbaum, Lawrason, Kaplan and Bittner (1944), Kirschbaum, Williams and Bittner (1946), Shimkin and Bryan (1943), Strong (1945), Orr (1946), demonstrated that carcinogenic hydrocarbons induce the appearance of breast cancer in females of low-cancer strains or accelerate the development of breast tumours in female mice of high-cancer strains. In these experiments it was also shown that oestrogenic influence is important in the induction of breast cancer by carcinogenic hydrocarbons, since mice developing these tumours in the absence of treatment with oestrogens were females, with one exception, a CBA male (Orr, 1943). The strains employed, the various routes by which the carcinogenic hydrocarbons were given and the results obtained by different workers are presented in Table I.

In connection with these observations, experiments were undertaken in order to ascertain the possibility of induction of breast cancer in mice of a high- and a low-cancer strain by treatment with oestrogenic hormones, methylcholanthrene, and combined application of oestrogens and methylcholanthrene.

MATERIAL AND METHODS.

C57 black low-breast-cancer strain females and males, and C3H high-breast-cancer strain males were used. The male mice of both strains, when 60–70 days old, were divided into three groups : mice of the first group received oestrone and methylcholanthrene, mice of the second group methylcholanthrene alone, and mice of the third group oestrone alone. C57 strain females, when 6 weeks old, were divided into two groups : mice of the first group were forcibly bred, mice of the second group were maintained as virgins ; both groups were treated with methylcholanthrene alone.

Oestrone was applied as a 0·02 per cent solution of keto-hydroxy-oestrone in alcohol, by painting the mice twice weekly for a period of 12 months. Methylcholanthrene was administered according to the method described by Orr (1946). Sixteen drops of 0·5 per cent solution in sweet almond oil were applied, at fortnightly intervals, to the skin of mice, four on each side of the dorsal and ventral surfaces of each animal.

The C57 females of both groups received fortnightly applications of methylcholanthrene from the age of 6 weeks till death or the appearance of a tumour.

In male mice the mammary gland is in a rudimentary state and they do not have nipples (Lacassagne, 1936). Administration of oestrogenic hormones leads to the progressive development of the mammary glands described in detail by Turner and Gomez (1934), Burrows (1935, 1936), Gardner (1935, 1939, 1941*b*), Lacassagne (1939*b*), Loeb (1940), Loeb and Suntzeff (1941). This process does not progress beyond hyperplasia of ducts and acini in male mice of strains in which breast cancer does not appear spontaneously. The nipples cannot be found, however, in male mice even after intensive treatment with oestrogens (Orr, 1943). Because of these observations and the findings that breast cancer can be induced by methylcholanthrene in males treated simultaneously with oestrogens (Orr, 1943), the C57 and C3H strain males were painted for 3 months with oestrone before the treatment with methylcholanthrene. After 3 months of painting with oestrone both the C57 and C3H males received their first application of methylcholanthrene which was then continued at fortnightly intervals until death or appearance of a tumour, except in the group of C3H males painted with oestrone which received only 14 applications of methylcholanthrene. All animals were examined once a week for the appearance of tumours.

RESULTS.

The results are shown in Tables II and III.

As can be seen from Table II, one breast tumour developed in C57 low-breast-cancer strain males treated with oestrone and methylcholanthrene. No tumours were induced in C57 males after treatment with oestrone or methylcholanthrene alone. Breast tumours developed in breeding and virgin C57 female mice which were treated with methylcholanthrene. The number of mice is too small for any conclusions to be drawn about the difference in the tumour incidence between

TABLE I.—*Influence of Hydrocarbons on the Development of Breast Cancer in Mice.*

Authors.	Hydrocarbon. Route.	Hydrocarbon. Dose.	Strain of mice.	Sex.	No. of mice.	No. of tumours.	Average tumour age (days).
Perry and Ginzton (1937)	Painting (twice weekly)	0·3% 1:2:5:6-dibenzanthracene in benzene + 0·1% theelin	Albino stock	F	65	17	180
Orr (1939)	Pellets	2% methylcholanthrene or 1:2:5:6-dibenzanthracene in paraffin	Outbred laboratory stock	F	215	4	—
Bonser and Orr (1939)	Sub-cutaneous	2 mg. methylcholanthrene (MC) in lard	IF	F (M)	32 (19)	12 (0)	..
			White label	F (M)	10 (5)	2 (0)	..
			Bagg albino	F	13 (6)	1 (0)	..
			CBA	F	10 (16)	2 (0)	..
Strong and Smith (1939)	Sub-cutaneous	1 mg. MC in 0·1 c.c. sesame oil	NH and JK	F	10	10	..
Mider and Morton (1939)	Painting (twice weekly)	0·5% MC in benzene	dba 212	F (M)	65 (75)	13 (0)	180 (—)
Strong and Williams (1941)	Sub-cutaneous	1 mg. MC in 0·1 c.c. sesame oil	NH	F (M)	384 (416)	45 (0)	160 (—)
Engelbreth-Holm (1941)	Painting (twice weekly)	0·5% MC in benzene	dba	F	19	19	255
Engelbreth-Holm and Lefevre (1941)	Painting or sub-cutaneous	0·5% 9:10-dimethyl-1:2-benzanthracene in benzene or 0·5–1 mg. in olive oil	dba	F	94	10	270
			AKa	F	34	0	..
Orr (1943)	Intranasal	0·5% MC in sweet almond oil	IF	vF (M)	26 (18)	18 (0)	..
			IF	M oestrogenised	16	11	..
			CBA	vF (M)	6 (16)	4 (1)	..

TABLE I—*continued*.

Authors.	Hydrocarbon. Route.	Dose.	Strain of mice.	Sex.	No. of mice.	No. of tumours.	Average tumour age (days).
Kirschbaum *et al.* (1944)	Painting (twice weekly)	0·5% MC in benzene	dba	F (vF)	27 (31)	27 (4)	105 (191)
			dba	M	48	..	..
Kirschbaum and Bittner (1945)	Painting 2–3 times weekly	0·25% MC in benzene	dba	F (vF)	27 (31)	27 (4)	110–145
			C3H	vF	12	..	..
			C3H × dba	F (vF)	6 (7)	5 (1)	110–145
			C3H × dba	F	16	1	200
Strong (1945)	Sub-cutaneous	1 mg. MC in 0·1 c.c. sesame oil	NHO	F	125	75	84–525
Kirschbaum *et al.* (1946)	Painting (three times weekly)	0·25% MC in benzene	Zb and Zb × dba	F (vF)	65 (34)	10	258 (260)
			NH and NH × dba	F	27	10	269
			C57	F	43	0	..
			Z and Z × dba	F (vF)	20 (20)	15 (1)	206 (240)
			dba and	F (vF)	34 (43)	30 (7)	186 (240)
			dba × Zb	M	48	0	..
			dba × NH	F	19	16	251
			A and A × dba	F	21	15	220
			dba × A	F	18	14	168
Orr (1946)	Cutaneous	0·5% MC in sweet almond oil	IF	vF	37	30	140
	Intra-peritoneal	0·5% MC in arachis oil	IF	vF	12	1	113

v = virgin.

TABLE II.—*Influence of Oestrone and Methylcholanthrene on the Development of Breast Cancer in C57 Low-Breast-Cancer Strain Mice.*

Treatment.	C57 Males.					C57 Females.					
	No. of mice.	Earliest tumour age (days).	No. of mice living to that age.	No. of tumours.	Average age at death (days).	No. of mice.	Earliest tumour age (days).	No. of mice living to that age.	No. of tumours.	Average tumour age (days).	Average age at death (days).
Oestrone	28	..	..	..	400	..	..	..	..	..	..
Methylcholanthrene	18	..	..	..	349	12b	294	10	1	..	285
						11v	307	6	2	343	296
Oestrone and methylcholanthrene	35	398	11	1	320	..	..	..	..	..	..

b = breeding females. v = virgin females.

TABLE III.—*Influence of Oestrone and Methylcholanthrene on the Development of Breast Cancer in C3H High-Breast-Cancer Strain Male Mice.*

Treatment.	No. of mice.	Earliest tumour age (days).	No. of mice living up to that age (days).	No. of tumours.	Average tumour age (days).	Average age of tumour-free mice at death (days).
Oestrone	31	174	14	8 (57%) SE = 17·5	316 SD = 44·2	248
Methylcholanthrene	22	..	..	..	..	253
Oestrone and methylcholanthrene	23	228	15	12 (80%) SE = 11·6	301 SD = 40·0	259

the breeding and the virgin females. Tumours were also induced in C3H high-breast-cancer strain males which were painted with oestrone alone, and with oestrone and methylcholanthrene combined (Table III). The percentage tumour incidence in males treated with oestrone alone does not significantly differ from that in males treated with oestrone and methylcholanthrene (D/S.E. = 1·4). There is no significant difference between the average tumour ages of C3H males of the two groups (D/S.E. = 0·8).

There was a considerable mortality among the experimental mice. The changes observed included loss of weight, retardation of body growth, atrophy of spleen and thymus, retention of urine, hydronephrosis. Two C57 males and one C57 female, which were treated with methylcholanthrene, developed general enlargement of lymphoid tissue, and in the C57 male which developed breast cancer a lymphosarcoma was found in the abdominal cavity. One C57 virgin female developed a spindle-celled sarcoma in the subcutaneous tissue.

Skin tumours were found in 10 out of 18 C57 males treated with methylcholanthrene alone and in 10 out of 35 C57 males treated with oestrone and methylcholanthrene. In C57 breeding females, 8 out of 12, and in C57 virgin mice, 3 out of 11, developed skin tumours. Skin tumours were also found in 4 out of 23 C3H males painted with oestrone and methylcholanthrene and in 8 out of 22 C3H males treated with methylcholanthrene. In 4 C3H males which showed skin tumours mammary tumours were also present. The skin tumours, however, were not examined histologically and so their malignancy has not been established.

All breast tumours were examined microscopically. The tumours induced by oestrone in C3H males resembled in every detail the mammary tumours which develop spontaneously in C3H female mice. Squamous metaplasia was observed in two tumours induced in C3H males by oestrone and methylcholanthrene. The breast tumours induced in C57 mice, both male and female mice, showed a much greater degree of squamous metaplasia than the breast tumours induced in C3H male mice. In some of the induced tumours, as in the case of spontaneous tumours, variations in structure between different parts of the same tumour were found. Sometimes the tumour cells were packed in solid sheets while elsewhere they occurred singly or in small groups surrounded by an abundant stroma; in some parts acinar structure could be clearly seen while in other parts it was lost entirely; in places elongated cords of spindle-shaped tumour cells were found. Metastases were observed only once, in one of the axillary glands of the tumour-bearing C57 male.

DISCUSSION.

Mammary tumours were induced in C3H high-breast-cancer strain males after cutaneous administration of methylcholanthrene combined with the application of an oestrogenic hormone. Treatment with the carcinogen alone failed to induce mammary cancer in C3H high- and C57 low-breast-cancer strain males. Combined administration of methylcholanthrene and oestrogen induced breast cancer in a C57 low-breast-cancer strain male. Thus the observations recorded by previous workers in this field were confirmed and the influence of hormonal factors on the development of carcinogen-induced breast cancer in mice shown.

Mammary tumours were also induced in C57 low-breast-cancer strain females, both breeding and non-breeding. This observation again confirmed the

results of other investigators. It is, therefore, possible to induce breast cancer in mice, both males and females, which lack the mammary tumour agent.

In the present experiments breast cancer was induced in mice of a sub-line of C57 black low-breast-cancer strain which has a low susceptibility to spontaneous breast cancer. Breast tumours developed in C57 black strain females, both breeding and non-breeding, after treatment with methylcholanthrene, and a C57 black strain male after combined treatment with oestrone and methylcholanthrene. It is not known whether the failure of Kirschbaum, Williams and Bittner (1946) to induce breast cancer in breeding C57 black strain females with methylcholanthrene was due to the different method of application of methylcholanthrene or to differences in susceptibility of the two sub-lines of the C57 strain. No tumours were observed in either C3H or C57 black strain males treated with methylcholanthrene alone. This supports the findings of other workers that hormonal factors play a part in the development of tumours induced by methylcholanthrene. Orr (1943), however, reported the induction of breast cancer in one out of 16 CBA low-breast-cancer strain males after intranasal administration of methylcholanthrene in the absence of treatment with oestrogen. He also obtained breast cancer in virgin mice of two low-breast-cancer strains (IF and CBA) after treatment with methylcholanthrene. In the present experiments tumours developed in two non-breeding C57 females treated with methylcholanthrene. Mider and Morton (1939) failed to induce breast cancer in non-breeding dba high-breast-cancer strain mice, while Strong and Smith (1939), Strong and Williams (1941) and Strong (1945) in their successful experiments on the induction of breast cancer in JK, NH and NHO low-breast-cancer strain mice employed breeding females only.

The present experiments confirm the findings of other workers that breast tumours can be induced in male mice of susceptible strains after treatment with oestrogenic hormones (Shimkin and Andervont, 1941, 1942; Bittner, 1941; Dmochowski and Gye, 1944; Bonser, 1944*a*, 1944*b*). The present findings also confirm the observation that mammary tumours develop in mice of resistant strains following treatment with methylcholanthrene. The C57 low-breast-cancer strain mice of the sub-line used, when foster-nursed soon after their birth by RIII high-breast-cancer strain females, develop an incidence of 11 per cent of breast tumours, but fail to develop breast cancer when given the tumour agent at the age of 4–6 weeks, even after large quantities of the agent and in spite of increased hormonal stimulation by repeated pregnancies (Dmochowski, 1948). Thus mature mice of this strain though resistant to the mammary tumour agent are susceptible to a different agent, i.e. methylcholanthrene. The treatment with oestrogenic hormones failed to induce breast cancer in C57 low-cancer strain males. This again confirms the previous findings (Twombly, 1939, 1940; Bittner, 1940, 1941; Shimkin and Andervont, 1941, 1942; Dmochowski and Gye, 1944) that an increased oestrogenic stimulation overcomes the threshold of low- or non-susceptibility and results in the development of breast tumours only in the presence of the mammary tumour agent.

Treatment with methylcholanthrene induced in C57 black low-cancer strain females, breast tumours which, although similar in their structure to spontaneous breast cancer of high- and of low-breast-cancer strain mice, show an increased incidence of squamous metaplasia. The tendency to squamous metaplasia was found in 2 out of 12 tumours induced in C3H males by the combined treatment

with oestrogen and methylcholanthrene and none was found among the 8 tumours in C3H males following treatment with oestrogen. These changes were much less pronounced than those found in methylcholanthrene-induced breast tumours of C57 black strain mice or IF strain mice (Orr, 1946). Kirschbaum, Williams and Bittner (1946) came to the conclusion that the histogenesis of methylcholanthrene-induced tumours is different from that of spontaneous tumours.

The development of breast cancer in mice of high- as well as of low-breast-cancer strains following treatment with carcinogenic hydrocarbons made the explanation of the origin of such tumours as a result of the oestrogen-like action of methylcholanthrene inadequate and made it necessary to determine whether any part was played by the mammary tumour agent in the development of methylcholanthrene-induced tumours, especially in low-breast-cancer strains. Experiments, which will be described in a later paper, were therefore undertaken in order to ascertain whether methylcholanthrene can induce the production of the mammary tumour agent—either by activating a dormant agent or by newly forming it.

SUMMARY.

Breast tumours developed in C3H high- and C57 black low-breast-cancer strain male mice following cutaneous administration of a 0·5 per cent solution of methylcholanthrene in almond oil, combined with painting with a 0·02 per cent solution of oestrone in alcohol. No tumours developed in males of these two strains following treatment with methylcholanthrene alone, while the application of oestrone gave breast tumours in C3H strain males, but not in C57 black strain males.

Mammary cancer developed in both breeding and non-breeding C57 black low-breast-cancer strain females after similar treatment with methylcholanthrene.

REFERENCES.

BITTNER, J. J.—(1936) *Science*, **84**, 162.—(1939) *Publ. Hlth. Rep.*, **54**, 1590.—(1940) *J. nat. Cancer Inst.*, **1**, 155.—(1941) *Cancer Res.*, **1**, 290.

BONSER, G. M.—(1940) *Amer. J. Cancer*, **38**, 319.—(1944*a*) *J. Path. Bact.*, **56**, 15.—(1944*b*) 'Rep. Brit. Emp. Cancer Campgn.,' p. 44.

Idem AND ORR, J. W.—(1939) *J. Path. Bact.*, **49**, 171.

BURROWS, H.—(1935) *Brit. J. Surg.*, **23**, 191.—(1936) *J. Path. Bact.*, **42**, 161.

DMOCHOWSKI, L.—(1948) *Brit. J. Cancer*, **2**, 94.

Idem AND GYE, W. E.—(1944) *Brit. J. exp. Path.*, **25**, 115.

DOBROVOLSKAÏA-ZAVADSKAÏA, N., AND ADAMOVA, N.—(1938) *Bull. Ass. franç. Cancer*, **27**, 308.—(1939) *Ibid.*, **28**, 76.

ENGELBRETH-HOLM, J.—(1941) *Cancer Res.*, **1**, 109.

Idem AND LEFEVRE, H.—(1941) *Ibid.*, **1**, 102.

GARDNER, W. U.—(1935) *Endocrinology*, **19**, 1656.—(1939) *Arch. Path.*, **27**, 1381.—(1941*a*) *Cancer Res.*, **1**, 345.—(1941*b*) *Endocrinology*, **28**, 53.

KIRSCHBAUM, A., AND BITTNER, J. J.—(1945) *Proc. Soc. exp. Biol., N.Y.*, **58**, 18.

Idem, LAWRASON, F. D., KAPLAN, H. S., AND BITTNER, J. J.—(1944) *Ibid.*, **55**, 141.

Idem, WILLIAMS, W. L., AND BITTNER, J. J.—(1946) *Cancer Res.*, **6**, 354.

LACASSAGNE, A.—(1936) *Amer. J. Cancer*, **27**, 217.—(1939*a*) *C.R. Soc. Biol., Paris*, **132**, 222.—(1939*b*) *Ergebn. Vitamin. Hormonforsch.*, **2**, 259.

Idem AND DANYSZ, S.—(1939) *C.R. Soc. Biol. Paris*, **132**, 395.

LOEB, L.—(1940) *J. nat. Cancer Inst.*, **1**, 169.
Idem AND SUNTZEFF, V.—(1941) *Arch. Path.*, **32**, 739.
MAISIN, J., AND COOLEN, M. L.—(1936) *C.R. Soc. Biol., Paris*, **123**, 159.
MIDER, G. B., AND MORTON, J. J.—(1939) *Proc. Soc. exp. Biol., N.Y.*, **42**, 583.
ORR, J. W.—(1939) *J, Path. Bact.*, **49**, 157.—(1943) *Ibid.*, **55**, 483.—(1946) *Ibid.*, **58**, 589.
PERRY, I. H., AND GINZTON, L. L.—(1937) *Amer. J. Cancer*, **29**, 680.
SHIMKIN, M. B.—(1945) In a Symposium on Mammary Tumours in Mice. By Members of the Staff of the Nat. Cancer Inst., Washington (Amer. Ass. Adv. Sc.), p. 85.
Idem AND ANDERVONT, H. B.—(1941) *J. nat. Cancer Inst.*, **1**, 599.—(1942) *Ibid.*, **2**, 611.
Idem AND BRYAN, W. R.—(1943) *Ibid.*, **4**, 25.
STRONG, L. C.—(1945) *Proc. Soc. exp. Biol., N.Y.*, **59**, 217.
Idem AND SMITH, G. M.—(1939) *Yale J. Biol. Med.*, **11**, 589.
Idem AND WILLIAMS, W. L.—(1941) *Cancer Res.*, **1**, 886.
TWOMBLY, G. H.—(1939) *Proc. Soc. exp. Biol., N.Y.*, **40**, 430.—(1940) *Ibid.*, **44**, 617.
TURNER, G. W., AND GOMEZ, E. T.—(1934) quoted by Dalton, H. J., in a Symposium on Mammary Tumours in Mice. By Members of the Staff of the Nat. Cancer Inst., Washington (Amer. Ass. Adv. Sci.), p. 39.

THE PERSISTENCE OF LATENT TUMOUR CELLS INDUCED IN THE MOUSE'S SKIN BY A SINGLE APPLICATION OF 9 : 10-DIMETHYL-1:2-BENZANTHRACENE.

I. BERENBLUM AND P. SHUBIK.

From the Oxford University Research Centre of the British Empire Cancer Campaign, Sir William Dunn School of Pathology, Oxford.

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IN a previous communication (Berenblum and Shubik, 1947) it was shown that the non-carcinogenic agent, croton oil, could elicit tumours in the mouse's skin following an interval of as long as 20 weeks after a single application of the carcinogen, 9:10-dimethyl-1:2-benzanthracene, and that the number of tumours produced was the same as that obtained when the interval was only 3 days. The evidence was considered as strong support in favour of the hypothesis that the carcinogen, as initiator, converts a small number of cells to "latent tumour cells," and that the latter persist unchanged until promoted by a further stimulus into morphological tumours.

The present investigation has been undertaken to determine the limits within which such a hypothesis remains tenable, by studying the effect of a time interval much longer than 20 weeks between the single application of the carcinogen and the commencement of croton oil treatment.

METHODS.

White, female mice of the Swiss strain, originating from the Medical Research Council and bred in this laboratory, were used. They were maintained throughout on an adequate diet, with water *ad lib.*, as in the previous investigations by the authors.

The experimental area of skin, in the interscapular region, was clipped periodically with fine scissors for removal of hair, and the test solutions applied with a fine glass rod. Medicinal liquid paraffin (light mineral oil) was used as solvent throughout.

EXPERIMENTAL.

A solution of 1·5 per cent 9:10-dimethyl-1:2-benzanthracene in liquid paraffin was applied once only to the skins of 25 mice, and after an interval of 43 weeks, during which the animals were inspected regularly but received no treatment of any sort, twice weekly applications of a 5 per cent solution of croton oil in liquid paraffin were begun, and continued for 17 weeks, at which time the experiment was concluded, owing to the departure from Oxford of the authors concerned.

For comparison, 4 groups of 25 mice each, of the same strain, and maintained similarly, received a single application of the carcinogen, followed after only a 3-week interval by twice-weekly applications of the croton oil solution. These mice formed part of another investigation by Berenblum and Shubik (1949).

In the experimental group 2 mice died during the 43-week interval, while one mouse developed a skin tumour at the 40th week, i.e. before the commencement of croton oil treatment. Of the remaining 22 mice, 9 developed tumours, at an average latent period of 9·1 weeks (Table I). In the 4 control groups

TABLE I.—*The Influence of Interval Between the Single Application of Carcinogen (DMBA) and the Croton Oil Treatment (twice weekly for 17 weeks).*

Series.	Interval.	No. mice used.	Survivors at time of 1st tumour.	Mice with tumours.	Percentage with tumours.	Average latent period (weeks). *
Expt. group	43 weeks	25	22	9	41	9·1
Control 1	3 ,,	25	25	12	48	10·0
,, 2	3 ,,	25	25	7	28	9·9
,, 3	3 ,,	25	25	17	68	10·2
,, 4	3 ,,	25	25	17	68	9·5
,, Total	3 ,,	100	100	53	53	9·9
Control of previous expt.†	3 days	92	62	35	56	9·2

* Latent period counted from commencement of croton oil treatment. For total latent period from commencement of experiments, add interval.

† From figures of Berenblum and Shubik (1947), Table III, series C, corrected for 17 weeks.

there was considerable variation in the tumour incidence, giving a standard deviation (σ) of 4·9 tumour-bearing animals per group of 25. The tumour incidence in the experimental group (9/22, or 41 per cent) is thus not significantly lower than in the control groups. In fact, a slight falling off in the tumour incidence might have been expected in mice that were 43 weeks older than their controls at the time of tumour development. The experimental results (41 per cent tumours ; 9·1 weeks average latent period) may also be compared with the control series of some earlier published results (58 per cent tumours ; 9·5 weeks average latent period), in which the interval was only 3 days.

DISCUSSION.

The earlier results (Berenblum and Shubik, 1947), which showed that the latent tumour cells induced by a carcinogen persisted for at least 20 weeks, has been questioned by Rusch and Kline (1948), who, while confirming the basic promoting activity of croton oil, maintained that with intervals of 3 or more months between the carcinogen and croton oil treatments, the tumour incidence decreased. Analysis of their published data reveals that the discrepancy between their results and those of Berenblum and Shubik lies in the method of presentation of data. Rusch and Kline failed to make allowance for the intervals in their calculations of latent periods, the comparisons of their various groups being made at set times dating from the first application of the carcinogen. For strict comparison between their group of carcinogen immediately followed by croton oil, and the group in which there was a 3-month interval, the croton oil treatment in the latter should have been continued for 3 months after the first experiment was completed, so that the total period of croton oil treatment was the same in both. This was not done, and consequently their conclusions are invalidated.

The present results, with an interval of 43 weeks (10 months) between the carcinogen and the croton oil treatment, confirms the original results of Berenblum and Shubik (1947), and lends further support to the view that the " latent tumour cells," induced by a carcinogen, represents a change which, to all intents and purposes, may be considered irreversible.

SUMMARY.

Croton oil, when applied 43 weeks after a single painting of the skin with 9:10-dimethyl-1:2-benzanthracene, elicits a tumour incidence similar to that occurring when the interval is 3 weeks or less.

The results add further support to the view that the " latent tumour cells," induced by a single application of a carcinogen, represent an irreversible change from normal cells.

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REFERENCES.

BERENBLUM, I., AND SHUBIK, P.—(1947) *Brit. J. Cancer*, **1**, 383.—(1949) *Ibid.*, **3**, 109.
RUSCH, H. P., AND KLINE, B. E.—(1948) *Proc. Soc. exp. Biol., N.Y.*, **69**, 90.

THE RELATIVE CARCINOGENIC ACTIVITY OF SIX ISOMERIC AMINOAZOTOLUENES.

H. G. CRABTREE.

From the Laboratories of the Imperial Cancer Research Fund, London, N.W.7.

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It has been suggested from studies of the action of inhibitors of S-metabolism on tumour induction by carcinogenic hydrocarbons that S-metabolism and the process of carcinogenesis are closely linked (Crabtree, 1947). This association is emphasized when the properties of azo-carcinogens are considered. The products of their metabolism have been shown to inhibit the activities of urease (Potter, 1942) and succinoxidase (Elson and Hoch-Ligeti, 1944), two enzymes dependent upon intact SH-groups for their proper functioning. Kensler, Dexter and Rhoads (1942) studied the relative inhibitory action of a series of " split-products " on a standard diphospho-pyridine-nucleotide system, and sought to correlate this activity with the carcinogenic power of the parent azo-compound. Suggestive parallelisms were found, but a clear-cut relationship did not emerge.

Much of this work has been based on the conception (experimentally verified for p-dimethylaminoazobenzene by Stevenson, Dobriner and Rhoads (1942), and for azo-benzene by Elson and Warren (1944)) that an azo-carcinogen is initially metabolized by the process of reductive fission with the liberation of an amine and a diamine, and that the latter is the effective carcinogen by virtue of its capacity to interfere with normal enzymic activities.

It is not clear why the diamine moiety alone should have been conceived as having special significance for the process of cancer induction. A survey of the azo-compounds so far tested for carcinogenic activity makes it evident that in some cases the amine moiety of the reduced molecule plays a significant role in determining potency, e.g. on comparing p-dimethylaminoazobenzene with its p′-methyl derivative, the introduction of the p′-methyl group causes a change from a very strong to a very weak carcinogen.

The present work was undertaken to probe this problem further. Isomeric aminoazotoluenes were chosen as suitable for a study of the relative contributions made by the amines and diamines which would arise from their reduction. The well-known carcinogen 4′-amino-2 : 3′-azotoluene (o : o) and the non-carcinogen 2′-amino-4 : 5′-azotoluene (p : p) served as bases of reference, and their behaviour was compared with that of the hybrid compounds o : p and p : o. Two other isomers—m : m and p : m—were also tested to provide further comparative data.

Note on abbreviations used.—All the isomeric aminoazotoluenes are prepared by coupling diazotized o-, m-, or p-toluidine with o-, m-, or p-toluidine, and the abbreviations reflect their molecular structure, e.g. p : m signifies that diazotized p-toluidine is coupled with m-toluidine, forming 4′-amino-4 : 2′-azotoluene, and so on.

MATERIAL AND METHODS.

Animals.—The experiments were carried out with rats of strain 52, descendants of a group originally selected for high susceptibility to transplantation of Jensen's rat sarcoma, and mice of the Simpson strain. A total of 210 rats weighing from 80 to 110 g., and 280 mice, 3–4 months old, were used, each divided into 7 groups of 30 rats or 40 mice. Six groups of each species were fed on the basal diet supplemented by 0·06 per cent of one of the six aminoazotoluenes, and the seventh group, fed on the basal diet alone, acted as controls.

In order that the possible influence of sex could be observed, each group of rats was divided into sub-groups of 10 males, 10 females, and 10 with members of both sexes. A similar separation was made with the mice into corresponding sub-groups of 10, 10 and 20.

Each cage or box contained 5 animals, and excess of the diets was always present, together with water *ad libitum*.

Diet.—The semi-synthetic diet was based on the one named R.D. 3 by Kirby (1947) which, in turn, closely resembled that developed by Miller, Miner, Rusch and Baumann (1941) as being suitable for promoting a high incidence of liver tumours with p-dimethylaminoazobenzene and related compounds. The latter was low in protein content, inadequate in some members of the B group of vitamins, and incapable of promoting normal growth in rats. The single source of carbohydrate was glucose monohydrate, for which Kirby substituted starch in his R.D. 3 diet. The carbohydrate used in the experiments described here was commercial dextrin, with 5 per cent sucrose added to increase palatability.

Basal diet.

	Per cent.
Casein (B.D.H. soluble, white, light)	12
Dextrin (B.D.H. technical yellow)	71
Sucrose	5
Salts (Harris, Krahl and Clowes, 1947)	4
Arachis oil	5
Cod-liver oil	1
Dried yeast	2

The dry constituents were mechanically mixed and the two oils then added with further mixing. The azo-dye was dissolved in the oils in an amount representing 0·06 per cent of the complete diet.

N.B.—When 0·06 per cent of p-dimethylaminoazobenzene was incorporated in this basal diet and fed to rats, liver tumours were produced in 90 per cent of the animals during 3–5 months.

Isomers of aminoazotoluene used.

Name of isomer.	Abbreviation.
2′-amino-4 : 5′-azotoluene	p : p
4′-amino-4 : 2′-azotoluene	p : m
4′-amino-3 : 2′-azotoluene	m : m
4′-amino-4 : 3′-azotoluene	p : o
2′-amino-2 : 5′-azotoluene	o : p
4′-amino-2 : 3′-azotoluene	o : o

Preparation of the aminoazotoluenes.

o : o.—Obtained commercially. Purified by crystallization from aqueous EtOH. Long, red-brown prisms with blue sheen. M.P. 100°.

m : m.—A solution of 13·8 g. (1 mole) $NaNO_2$ was added slowly to 57·4 g. (2 moles) m-toluidine hydrochloride dissolved in 200 ml. water, at room temperature (Mehner, 1902). The crystalline hydrochloride suspended in EtOH was neutralized with ammonia, and an equal volume of boiling water added. The free base was re-crystallized from aqueous EtOH. Golden yellow needles. M.P. 81°.

p : p (*a*) 4 : 4′-*diazoaminotoluene.*—Prepared by a modification of the method of Nietzki (1877). 96·6 g. (2 moles) p-toluidine hydrochloride were dissolved in 350 ml. H_2O and cooled to 5–7°. A solution of 23·4 g.(1 mole) of $NaNo_2$ was slowly run in over 2 hours with stirring. The pale yellow, microcrystalline diazoamino-compound was used for stage (*b*) without further purification. M.P. 114–115°. Crystallization from aqueous EtOH gave large prisms. M.P. 116°.

(*b*) 2′-*amino*-4 : 5′-*azotoluene.*—(p : p) 1 mole (*a*) + 1 mole p-toluidine hydrochloride + 6 moles p-toluidine were heated at 65° for 10 hours (Zincke and Lawson, 1886). The molten mass was neutralized with dilute NaOH, p-toluidine removed by steam distillation, and the brown residue crystallized from aqueous EtOH. Orange red needles. M.P. 119°.

p : m.—67·5 g. 4 : 4′-diazoaminotoluene (1 mole) + 43·05 g. m-toluidine hydrochloride (1 mole) + 450 ml. EtOH were kept at 2° for 3 days, with occasional shaking (Mehner, 1902). The crystalline dye-hydrochloride was filtered (35 g.) and a second crop (19 g.) was obtained after addition of 500 ml. N HCl and standing overnight at 2°. Recrystallization from aqueous EtOH gave orange-red needles with metallic sheen. The base was obtained as described for m : m. Large rectangular golden-yellow plates. M.P. 128°.

p : o.—Prepared by method used for p : m (Mehner, 1902). After 5 days at 2° the red-brown solution was treated with an equal volume of N HCl, and EtOH largely removed on the water bath. The dye-hydrochloride crystallized after standing overnight at 2°. Yield = 66 per cent theory. The base was obtained as described for m : m. Orange-red needles. M.P. 128°.

o : p (*a*) 2 : 2′-*diazoaminotoluene.*—Based on the method of Fischer and Wimmer (1887). 28·7 g. o-toluidine hydrochloride (2 moles) + 8·7 ml. HCl (S.G. 1·18 — 1 mole) + 100 ml. H_2O were cooled to − 5°, and a solution of 6·9 g. $NaNO_2$ (1 mole) added during 5 minutes. A solution of 25 g. sodium acetate was slowly run in, and, after stirring for 4 hours, the pale yellow, micro-crystalline diazoamino-compound was collected. Yield = 70 per cent theory. M.P. 47°. This was used for (*b*) without further purification.

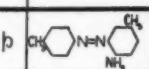
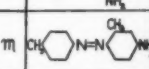
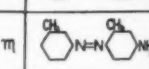
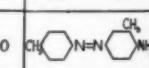
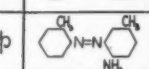
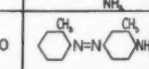
(*b*) 45 g. (*a*) (1 mole) + 28·7 g. p-toluidine hydrochloride (1·1 moles) + 380 ml. EtOH were kept at 2° overnight. 300 ml. N HCl were added and EtOH largely removed on the water bath. Yield of dye hydrochloride was 47 g. (90 per cent theory). Recrystallization from aqueous EtOH gave orange-red needle clusters. The base was obtained as described for m : m. Orange-red rectangular plates. M.P. 95°.

EXPERIMENTAL RESULTS.

The livers, and sometimes other organs, of all animals were examined at death, or whenever possible when death was imminent.

Many rats were lost through cannibalism and intercurrent infection, principally endemic bronchopneumonia. Mice responded badly to the basal diet, with or without the addition of dye. Many were lost in the first 6 months of the experiment, but the death-rate was lowered in the later stages by feeding them with standard laboratory food and the synthetic diet alternately for periods of two weeks.

TABLE I.—*Changes Produced by Six Isomeric Aminoazotoluenes in the Livers of Rats and Mice, surviving 300–595 days. The histological classification is based on a succession of changes reflecting increasing degrees of liver damage, and each × refers to the liver of one animal.*

		RAT LIVERS					MOUSE LIVERS				
	AMINOAZOTOLUENE ADDED TO BASIC DIET	NORMAL	MULTIPLE AREAS OF PERILOBULAR NECROSIS	REGENERATION OF LIVER CELLS IN NECROTIC AREAS	MICROSCOPIC NODULES OF HEPATOMA IN SOME AREAS OF REGENERATION	FULLY DEVELOPED HEPATOMA (ONE CHOLANGIOMA)	NORMAL	IRREGULARITY OF PATTERN AND SIZE OF NUCLEI	MORE SEVERE CHANGES WITH GIANT VACUOLATED NUCLEI	MICROSCOPIC NODULES OF HEPATOMA OR BEGINNING OF CHOLANGIOMA	MALIGNANT HEPATOMA (OCCASIONAL CHOLANGIOMA)
p:p		XXXXX XXXXX XXXXX	XXXXX X				XXXXX XXX	XXXX			
p:m		XXXXX XXXXX XXXXX XX	XXX					XXX	XXX	X	XXXXX
m:m		XXXXX XXXXX	XXXXX XXXX				XXXX		XXXXX XXXXX	X	
p:o		XXXXX XXXXX XXXXX	XXXXX XXX	X			XXXXX	XXXXX XXX	X	X	
o:p			XXX	XXXXX XXXXX X	XXX				XX	XXXXX X	XXX
o:o		XXXX	XXXXX XXX	XXXXX XX	XXXXX	XXX			XX	XXX	XXXXX XX
	BASIC DIET ALONE	XXXXX XXXXX XXXXX X	XXXXX XXX				XXXXX XXXXX X	XXXXX XX			

Under these circumstances quantitative estimates of the relative tumour-inducing properties of the various aminoazotoluenes were not easy to make. However, since the survival rates in the different groups of animals were not widely different, a qualitative picture of the broad features of the results emerged, and some significant differences in the action of the six isomeric compounds were demonstrated.

(a) *Histological observations.*

Omitting animals which died before 250 days, the average time of survival of both rats and mice in the various groups was 400–435 days from the beginning of the experiment. A few were alive after 595 days and were killed.

The whole liver was removed, fixed in Bouin and sections made from several or all of the lobes, and stained with eosin and haematoxylin. This was necessary, since macroscopically visible tumours were rare.

The histological findings are collected in Table I, where each × refers to the liver of one animal. The succession of changes used in this classification reflect increasing degrees of liver damage. The first characteristic abnormalities visible in the livers of rats differed from those commonly observed in the livers of mice, though the final pictures of tumour emergence were similar in both species. In building up the general picture four grades of cellular change have been used.

In rat livers perilobular necrosis constituted the primary change, and this was followed by regeneration of liver cells in the necrotic areas. In some regions of regeneration microscopic nodules of hepatoma made their appearance, and later these became visible to the eye as dull grey patches on the surface of the lobes. Often a simultaneous intense proliferation of bile ducts was manifest in one or more lobes, and the early phases of cholangioma formation would coincide with the development of small hepatomas. No metastases were ever found.

In mouse livers no preliminary necrosis was observed, but the cellular pattern became abnormal with great irregularity in the disposition and size of the nuclei. Further distortion was accompanied by the formation of cells with giant vacuolated nuclei, which preceded the emergence of microscopic hepatomas. The fully developed hepatomas of mice were more malignant than those of rats as judged by the degree of atypical growth, though again no metastases were ever found.

It will be noted that the earliest histological changes mentioned sometimes occurred in both rats and mice consuming the basal diet without dye-addition. This response of the liver to diets inadequate in several essential food factors may well reflect the basic disturbance of liver function which is a necessary precursor to the carcinogenic action of azo-compounds.

(b) *Relative carcinogenic activity of the six isomeric aminoazotoluenes.*

Considering the data summarized in Table I from the point of view of the relative carcinogenic potency of these isomers, three points emerge:

1. Mice were more susceptible than rats to the action of these azo-compounds. With the exception of p : p, which proved innocuous in both species, all the isomers were capable of inducing liver tumours in mice, but varied in potency among themselves. In three cases, o : o, o : p and p : m, malignant hepatomas were produced, and severe lesions, with one microscopic hepatoma, were caused by m : m.

2. Only two isomers—o : p and o : o—induced tumours in rats. In all other cases, except for one rat receiving p : o, perilobular necrosis represented the utmost limit of damage, and this lesion was found, though in lesser degree, in the livers of rats fed on the basic diet alone.

3. Except in the case of p : m, which induced a high percentage of tumours in mice surviving 400 days and was innocuous for rats, the relative potency of the isomers was similar in both species.

(c) *Growth rate of rats on basal diet with azo-dye addition.*

All rats (but not mice) were weighed at fortnightly intervals throughout the experiment. Small differences of growth-rate within the various groups became noticeable after 3–4 months, and later they became more accentuated. Since all other environmental factors were similar for all the groups, these differential growth rates could only be attributed to the azo-dyes themselves.

The possible influence of sex on the growth rate was assessed by keeping the rats of each series separated into three sub-groups containing respectively 10 males, 10 females and 5 males + 5 females. Though the average female rat weighed 15–20 g. less than the average male of the same age, the differential growth-rates due to the added azo-dyes were evident in both sexes, and were unaffected by pregnancies, since breeding did not occur after the first few months of the experimental period.

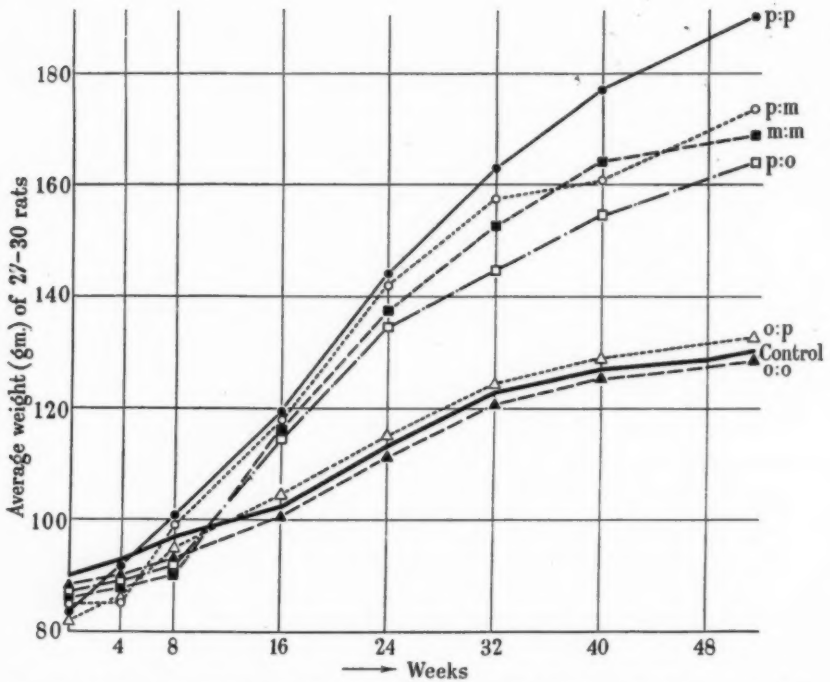


FIG. 1.—Growth curves of rats fed on the basal diet, with addition of 0·06 per cent of an aminoazotoluene. The differential effects on growth of the six isomers are shown.

The points shown in the curves in Fig. 1 are therefore based on the average weights of each group of rats, regardless of sex. The following features of these growth-curves may be emphasized :

1. The basal diet was inadequate for normal growth, but permitted limited growth and long survival.

2. The two isomers o : o and o : p had no inhibitory action on body growth, or more exactly, they had no additional effect on growth already inhibited by the basal diet.

3. The four remaining isomers—p : p, p : m, m : m and p : o—caused a stimulation of growth-rate in varying degrees, the most effective being the p : p isomer.

On comparing these effects on rat-growth with the histological data in Table I a suggestive correlation is obvious; all the isomers which caused growth-stimulation were non-carcinogenic, whereas those lacking this property were carcinogenic, for rats.

(d) *Growth rate of rats on basal diet, with addition of toluidines.*

Two investigations have shown that azo-compounds are metabolized in the rat by initial reduction at the azo-group, yielding the corresponding amines (Stevenson, Dobriner and Rhoads, 1942; Elson and Warren, 1944). On the assumption that this splitting process occurred with the aminoazotoluenes used

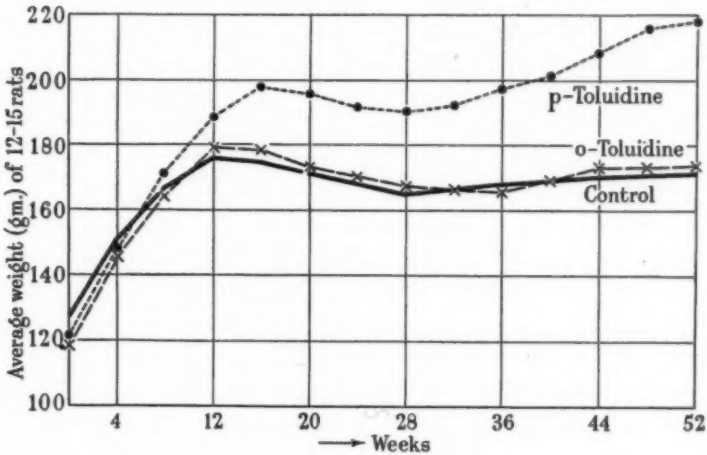


FIG. 2.—Growth curves of rats fed on the basal diet, with addition of 0·06 per cent o- or p-toluidine.

in the work reported here, the two carcinogenic isomers would yield o-toluidine, while 3 of the 4 growth-stimulating isomers would yield p-toluidine as the primary products of reductive fission.

The possible effects on growth of these two toluidines were therefore tested.

Forty-five rats of the 52 strain, each weighing 110–130 g., were divided into three groups of 15. Two groups were fed on the basal diet containing 0·06 per cent of either o-toluidine or p-toluidine, and the third group received the basal diet alone. Each group of 15 rats was subdivided into three separated groups of 5 males, 5 females, and 5 containing males and females. In the latter group two litters were born during the first 3 months, but none subsequently. They were weighed at fortnightly intervals over a year.

The growth curves are shown in Fig. 2. Since no difference of response due to sex was noted, the average weights refer to all surviving animals. At least 12 animals survived in each group for a year, but the general condition of those receiving p-toluidine was significantly better than that of the other groups.

The growth-response caused by o-toluidine was negligible, but p-toluidine acted as a growth-stimulator, particularly in the later stages of the experiment. The sag in the middle range of the curves is an odd feature for which no explanation can be offered, but it does not detract from the main result of this experiment.

Observations on these results.

This commentary will be restricted to the experiments in which rats were used. It makes comparison with previous work easier, since most investigations have been carried out on rats living under a standardized dietary regime similar to the one used here, and current hypotheses as to the mode of action of azo-carcinogens are largely based on such findings.

Though the degree of liver damage and the incidence of liver tumours was higher in mice than in rats, there is a broad parallelism between the relative potency of this series of aminoazotoluenes in the two species.

The variable response of different species to a given carcinogen remains a fundamental problem which cannot, as yet, be expressed in biochemical terms. Kirby (1945), considering the mechanisms by which the azo-compounds are degraded in the organism, has suggested that the known alternative paths of metabolism may vary quantitatively in rats and mice, with formation of benzidine derivatives (postulated by Cook, Hewitt, Kennaway and Kennaway, 1940) as the principal reaction occurring in mice, and reductive fission predominating in rats. On the other hand, Miller and Miller (1947) emphasize the importance of the protein constitution of the host, and associate the likelihood of tumours developing with the degree to which an azo-compound is " bound " to the proteins of rat livers. In the case of rats consuming p-dimethylaminoazobenzene this fixation is prominent, but does not occur in species resistant to the carcinogenic action of the substance. These differences can only be attributed to the special properties of the proteins characteristic for each species.

The " split-product " hypothesis.

Kirby (1945) has reviewed the work bearing on this hypothesis of the mechanism of carcinogenesis by azo-compounds, and throws doubt on its validity by citing several examples which fail to conform with its main premises. He emphasized in particular the low carcinogenic power of p′-methyl-p-dimethyl-aminoazobenzene when compared with the high activity of the parent p-dimethyl-aminoazobenzene, since both compounds should yield the same postulated active diamine split-product. This example, and others to be mentioned, makes it clear that the other half of the reduced molecule—the amine moiety—plays an important role in determining carcinogenic activity if the primary assumption be accepted that reductive fission is an essential preliminary to this process.

This consideration is emphasized by the results presented here. In fact the possible significance of the " second half " of the reduced azo-molecule, almost entirely ignored by previous workers, prompted these experiments. o : o was known to be carcinogenic, and p : p non-carcinogenic for rats and mice, or translated in terms of the " split-product " hypothesis, 2 : 5-toluylene diamine is active while 3 : 4-toluylene diamine is innocuous.

To throw light on the relative contributions made by the amine to diamine parts of the reduced molecule, the hybrid isomers o : p and p : o were prepared and their action compared with that of the related o : o and p : p isomers. The experiments demonstrated that o : o and o : p were carcinogenic, while p : p and p : o were non-carcinogenic.

Reviewing these results in the light of the " split-product " hypothesis, no correlation between carcinogenic activity and the potential diamine fission products is found. p : p and o : p should yield the same " innocuous " o-toluylene diamine, while o : o and o : p should yield the same " active " p-toluylene diamine, but the facts are otherwise, since each of these pairs contains one carcinogen and one non-carcinogen.

By contrast, when the amine halves of the reduced molecule are considered, the results show that the two isomers which should yield p-toluidine on reductive fission—p : p and p : o—are non-carcinogenic, while the pair yielding o-toluidine —o : o and o : p—are carcinogenic. In brief, if carcinogenic activity is indeed a consequence of the action of the " split-products," it is the amine, and not the diamine, which determines the subsequent biological response.

The literature contains several examples of the controlling influence which a methyl group exerts on carcinogenesis, when attached at a para position with respect to the azo-group. Miller and Baumann (1945) compared the activities of the o′, m′, and p′ methyl derivatives of p-dimethylaminoazobenzene with that of the parent compound. The m′-compound was extremely active (7 rats out of 8 bearing liver tumours in 4 months), the o′-compound moderately active (4 rats out of 9 bearing tumours in 8 months), and the p′-compound was very weakly carcinogenic (1 rat out of 10 with a tumour in 10 months). Nagao (1941) also found that p′-methyl-p-dimethylaminoazobenzene was only a weak carcinogen, and that the substances formed by placing a further methyl group in the ring carrying the dimethylamino-group were entirely innocuous. Sugiura (1948) tested a series of derivatives of p-methylaminoazobenzene containing an additional methyl group in the o′, m′ or p′ positions. The order of potency—m′>o′>p′—was similar to that mentioned above.

All these observations seem relevant to the present work. The common feature is the great loss of tumour-producing activity which results when a methyl group is added in the p′ position to the parent carcinogenic molecule, forming derivatives with the potentiality of liberating p-toluidine on reductive fission.

A working hypothesis is put forward tentatively in an attempt to co-ordinate the data outlined above. When the structural features and the biological action of these six isomeric azotoluenes are considered, a correlation is evident between their growth-stimulating properties, their lack of carcinogenic power, and the presence of an additional methyl group in the position described. The two latter characteristics are common to all the substances used by Miller and Baumann, Nagao and Sugiura, but these workers do not mention any differential growth responses in the rats used in their studies.

The amines emerging from reductive fission may undergo further metabolic changes (Stevenson, Dobriner and Rhoads, 1942 ; Elson and Warren, 1944). They are detoxicated and excreted, in the examples given, as acetylated diamines or as aminophenols. The breakdown products of the aminoazotoluenes have not yet been investigated, but presumably the toluidines are initially formed. Jaffe

and Hilbert (1888) fed the three isomers of acetotoluidine to dogs. o-Acetotoluidine was oxidized to an aminophenol and isolated as methylbenzoxalone, while m- and p-acetotoluidines were excreted as acetylated amino-benzoic acids. It may therefore be conjectured that all azo-compounds containing a methyl group at a position para to the azo-group will yield p-aminobenzoic acid as a final product of their metabolic degradation within the rat.

The experiment pictured in Fig. 2 shows that p-toluidine can stimulate growth in rats fed on a diet permitting only limited growth and maintenance. If this is due to the liberation of p-aminobenzoic acid, the question arises : Is the synthesis of folic acid indirectly influenced by feeding appropriate azo-compounds *via* the succession of changes

azo-compound ⟶ p-toluidine ⟶ p-aminobenzoic acid ⟶ folic acid ?

And further, what is the relation of folic acid to the induction of tumours ?

Folic acid, in its relation to the cancer problem, has been widely investigated in recent years, but its possible association with tumour induction is uncertain, since the major effort has been made in assessing its therapeutic possibilities. The early work of Lewisohn, Leuchtenberger, Leuchtenberger and Keresztesy (1946), who reported regression in a high percentage of transplanted mammary tumours when pteroyl triglutamic acid was injected intravenously, has been followed by several clinical trials (Leading Article, 1948). It has also been shown that the development of Rous sarcoma, after injection of the virus in baby chicks, was completely prevented by maintaining the birds on a folic-acid-free diet. Tumour growth was prevented in 40 per cent of birds up to 6 weeks of age. Certain folic acid antagonists were equally effective for this purpose, and were also able to cause cessation of growth in Rous sarcomas already established (Little, Sampath, Paganelli, Locke, and Subbarow, 1948).

In the field of bacterial metabolism there is strong evidence that p-aminobenzoic acid is concerned with the synthesis of folic acid, purines, thymine and methionine, and that the sulphonamides may inhibit reactions leading to the synthesis of proteins and nucleic acids (Woods, 1947).

The folic acid requirements of rats have not been studied so widely as those of chicks, since the symptoms of deficiency are not so easily recognized. Woolley (1947) states that " experimental tricks such as the administration of drugs are necessary to produce a dietary need for the vitamin." This is exemplified by the haematological response following the administration of sulphonamides to rats which is reversed by injection of folic acid (Endicott, Daft and Ott, 1945), or the similar changes produced by protein depletion or inadequate riboflavine (Kornberg, Daft and Sebrell, 1945).

It is tempting to interpret the experimental results described in this paper in terms of folic acid balance. The diamines resulting from the reductive fission of azo-carcinogens have been shown to inhibit many enzyme reactions under oxidative conditions, and it is conceivable that they interfere with the function of enzymes concerned in the synthesis of folic acid, producing adverse effects comparable to those produced by sulphonamides, though by a different mechanism. Conversely the non-carcinogenic, growth-stimulating isomers of amino-azotoluene could be visualized as precursors of p-aminobenzoic acid, the liberation of which could counteract the damage produced by depletion of folic acid.

The limited growth conditioned by a diet low in proteins and inadequate in its complement of B-vitamins reinforced by the postulated inhibition of folic acid synthesis would then constitute the metabolic disturbances favourable for the emergence of tumours.

SUMMARY.

1. Six isomeric aminoazotoluenes have been tested for their carcinogenic activity in the livers of mice and rats, when added at 0·06 per cent concentration to a standard, semi-synthetic diet. They are labelled o : o, m : m, p : p, o : p, p : o and p : m respectively to denote the two toluidines used in their preparation.

2. A series of characteristic histological changes, reflecting increasing degrees of liver damage, has been used in classifying the results, which serve to indicate the relative potency of the six isomers.

3. Mice were more susceptible than rats, though the order of potency, with one exception, was the same in both species. All the isomers, except p : p, proved carcinogenic for mice, but only two of them—o : o and o : p—were carcinogenic for rats.

4. The growth-curves of each group of rats consuming the different aminoazotoluenes are shown. The two carcinogens, o : o and o : p, did not influence the growth rate, but the four non-carcinogens—p : o, p : p, m : m and p : m—stimulated growth in varying degrees the p : p isomer being the most effective.

5. Rats were fed on the same basal diet containing 0·06 per cent of either o- or p-toluidine. o-Toluidine produced no effect on growth, but p-toluidine acted as a growth stimulant.

6. These results are discussed in the light of the " split-product " hypothesis, which they fail to support. Also, on the assumption that carcinogenic properties and growth effects are conditioned by their " split-products," a correlation had been established between growth stimulation, non-carcinogenicity and the release of p-toluidine.

7. Since the detoxication of p-toluidine can produce p-aminobenzoic acid, the possible relation between folic acid metabolism and the mechanism of cancer induction is considered.

I am greatly indebted to Dr. E. Vazquez Lopez for help and guidance in the histological aspects of this work. The method of classification shown in Table I was suggested by him, and, in assessing the degree of liver damage, his judgment was final.

REFERENCES.

Cook, J. W., Hewitt, C. L., Kennaway, E. L., and Kennaway, N. M.—(1940) *Amer. J. Cancer*, **40**, 62.
Crabtree, H. G.—(1947) *Brit. med. Bull.*, **4**, 345.
Elson, L. A., and Hoch-Ligeti, C.—(1944) *Biochem. J.* (Proceedings), **38**, x.
Idem and Warren, F. L.—(1944) *Ibid.*, **38**, 217.
Endicott, K. M., Daft, F. S., and Ott, M.—(1945) *Arch. Path.*, **40**, 364.
Fischer, B., and Wimmer, H.—(1887) *Berichte*, **20**, 1581.
Harris, P. N., Krahl, M. E., and Clowes, G. H. A.—(1947) *Cancer Res.*, **7**, 162.
Jaffe, M., and Hilbert, P.—(1888) *Z. Physiol. Chem.*, **12**, 295.
Kensler, C. J., Dexter, S. O., and Rhoads, C. P.—(1942) *Cancer Res.*, **2**, 1.
Kirby, A. H. M.—(1945) *Ibid.*, **5**, 683.—(1947) *Ibid.*, **7**, 333.

KORNBERG, A., DAFT, F. S., AND SEBRELL, W. H.—(1945) *Arch. Biochem.*, **8**, 431.
Leading article, (1948) *Brit. Med. J.*, ii, 827.
LEWISOHN, R., LEUCHTENBERGER, C., LEUCHTENBERGER, R., AND KERESZTESY, J. C.—(1946) *Science*, **104**, 436.
LITTLE, P. A., SAMPATH, A., PAGANELLI, V., LOCKE, E., and SUBBAROW, Y.—(1948) *Trans. N.Y. Acad. Sci.* **10**, 91.
MEHNER, H.—(1902) *J. prakt. Chem.*, **65**, 401.
MILLER, E. C., AND MILLER, J. A.—(1947) *Cancer Res.*, **7**, 468.
MILLER, J. A., AND BAUMANN, C. A.—(1945) *Ibid.*, **5**, 227.
Idem, MINER, D. L., RUSCH, H. P., AND BAUMANN, C. A.—(1941) *Ibid.*, **1**, 699.
NAGAO, N.—(1941) *Gann*, **35**, 8.
NIETZKI, R.—(1877) *Berichte*, **10**, 662.
POTTER, V. R.—(1942) *Cancer Res.*, **2**, 688.
STEVENSON, E. S., DOBRINER, K., AND RHOADS, C. P.—(1942) *Ibid.*, **2**, 160.
SUGIURA, K.—(1948) *Ibid.*, **8**, 141.
WOODS, D. D.—(1947) *Ann. Rev. Biochem.*, **16**, 613.
WOOLLEY, D. W.—(1947) *Ibid.*, **16**, 372.
ZINCKE, T., AND LAWSON, A. T.—(1886) *Berichte*, **19**, 1452.

PREVENTION OF ABDOMINAL FIBROIDS INDUCED WITH ARTIFICIAL OESTROGENS.

S. BRUZZONE.

From the Department of Experimental Medicine, National Health Service of Chile, Santiago.

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ABDOMINAL fibroids induced in the guinea-pig by α-oestradiol (Nelson, 1937; Lipschutz and Iglesias, 1939) are prevented when progesterone or other 3-keto-steroids are administered simultaneously with the oestrogen (Lipschutz and Vargas, 1941; Lipschutz, 1944; Lipschutz, Iglesias, Bruzzone, Fuenzalida and Riesco, 1948). The antifibromatogenic action of progesterone becomes manifest with as little as 13 to 24 μg. per day absorbed from a subcutaneously implanted tablet (Lipschutz, Bruzzone and Fuenzalida, 1944). Since similar abdominal fibroids have been elicited also with diethylstilboestrol and hexoestrol (Lipschutz and Vargas, 1940; Lipschutz, Vargas, Egaña and Bruzzone, 1941) the question arose whether progesterone will be as active against these artificial oestrogens as against α-oestradiol. This question is of considerable interest. Nothing definite is so far known on the mechanism by which the antifibromatogenic action of steroids is effected. Will the greater resistance of these artificial oestrogens against intrahepatic inactivation (Zondek, Sulman and Sklow, 1943; Segaloff, 1944; Lipschutz, Quintana and Bruzzone, 1944) be associated with a quantitatively different behaviour towards progesterone?

EXPERIMENTS.

Tablets containing 40 per cent of diethylstilboestrol or hexoestrol mixed with 60 per cent of cholesterol were implanted subcutaneously. At the same time a tablet of pure progesterone or a tablet containing 40 per cent of progesterone was implanted. Absorption from a similar mixture is non-selective, the concentration of the specific steroid remaining unaltered (Shimkin and White, 1941; Fuenzalida, 1944); this refers also to diethylstilboestrol. To obtain absorption of variable quantities of progesterone half a tablet or less was implanted in several animals. Results with 96 castrated female guinea-pigs are given in Table I.

TABLE I.—96 *Female Guinea-pigs; Necropsied at* 90 *Days of Treatment.*

Number of animals.	Oestrogen per day. (μg.*)	Progesterone per day. (μg.*)	Weight of uterus. (g.)	F.T.E. Units.†	Frequency of tumours class 2 and 3.†
	Stilboestrol				
15	6–20	0	4·9	4·9	1·4
14	4–20	2–9	4·1	3·7	1·0
10	11–17	10–18	4·2	3·7	1·2
3	8–9	*36–41*‡	3·7	1·5	0
3	7–9	60–70‡	2·3	1·3	0·7
6	7–9	85–104‡	2·6	1·8	0·3
15§	9–21	*120–305*‡	2·1	0·9	0·1
	Hexoestrol				
5	3–10	0	3·9	4·3	1·4
8	4–10	10–17	3·9	2·4	0·5
2	2–9	*22–34*	2·6	1·3	0
15§	8–13	*122–261*‡	1·7	0·4	0

* Calculated from the loss of weight of the tablet divided by number of days, on the assumption of non-selective absorption.

† F.T.E. = Fibrous Tumoural Effect; units according to Lipschutz and Vargas (1939) and Lipschutz and Maass (1944).

‡ Tablets of pure progesterone or part of a tablet. In the remaining groups—tablets containing 40 per cent of progesterone.

§ From experiments of E. Peña (1943).

There is no doubt that the fibromatogenic action of both diethylstilboestrol and hexoestrol has been prevented by progesterone; with quantities of 120 μg. or more per day the preventive action of progesterone was as pronounced as in former experiments with α-oestradiol (Fig. 1).

There was certainly a preventive action also with quantities less than 120 μg. and even with as little as 36 to 41 and 22 to 34 μg. respectively, as shown both by the diminution of uterine weight and the diminution of the tumoural reaction. Prevention was, indeed, at these levels less complete. But these figures of active quantities of progesterone are not very distant from those obtained in our former work with the antifibromatogenic action of progesterone against α-oestradiol. There is also the fact that statements about absorption per day are stultified by its not being uniform throughout the experiment on account of the diminished surface, and by substances entering into the tablet from outside; errors are

consequently considerable (Folley, 1944). This is why it would be daring to interpret the present results as indicative of a greater resistance of artificial oestrogens to the antifibromatogenic action of progesterone.

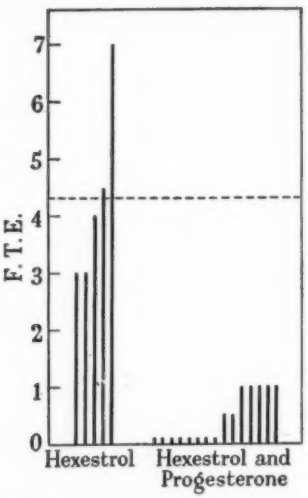

FIG. 1.—Antifibromatogenic action of progesterone (122 to 261 μg. per day) in experiments with hexoestrol. Horizontal line—average F.T.E. with hexoestrol alone.

SUMMARY.

The fibromatogenic action of diethylstilboestrol and hexoestrol is prevented by the simultaneous administration of progesterone as is the fibromatogenic action of α-oestradiol. The quantity of progesterone necessary for preventing the fibromatogenic action of these artificial oestrogens, though somewhat greater than in experiments with α-oestradiol, was not far from that.

REFERENCES.

FOLLEY, S. J.—(1944) *Proc. Roy. Soc.*, B, **132**, 142.
FUENZALIDA, F.—(1944) *Rev. Canad. Biol.*, **3**, 366.
LIPSCHUTZ, A.—(1944) *Nature*, **153**, 260.
Idem, BRUZZONE, S., AND FUENZALIDA, F.—(1944) *Cancer Res.*, **4**, 179.
Idem AND IGLESIAS, R.—(1939) *C.R. Soc. Biol., Paris*, **129**, 519.
Iidem, BRUZZONE, S., FUENZALIDA, F., AND RIESCO, A.—(1948) *Acta Unio Int. Cancer*, **6**, 85.
Idem AND MAASS, M.—(1944) *Cancer Res.*, **4**, 18.
Idem, QUINTANA, U., AND BRUZZONE, S.—(1944) *Proc. Soc. exp. Biol. N.Y.*, **55**, 43.
Idem AND VARGAS, L.—(1939) *C.R. Soc. Biol., Paris*, **131**, 27.—(1940) *Lancet*, i, 541.—(1941) *Endocrinology*, **28**, 669.
Iidem, EGAÑA, E., AND BRUZZONE, S.—(1941) *Proc. Soc. exp. Biol. N.Y.*, **48**, 467.
NELSON, W. O.—(1937) *Anat. Rec.*, **68**, 99.
PENA, E.—(1943) M.D. Thesis, Universidad de Chile.
SEGALOFF, S.—(1944) *Endocrinology*, **34**, 335.
SHIMKIN, M. B., AND WHITE, J.—(1941) *Ibid.*, **29**, 1020.
ZONDEK, B., SULMAN, F., AND SKLOW, J.—(1943) *Ibid.*, **33**, 333.

THE EFFECTS OF THIOUREA ON THE DEVELOPMENT OF SPONTANEOUS TUMOURS ON MICE.

E. VAZQUEZ-LOPEZ.

From the Laboratories of the Imperial Cancer Research Fund, London, N.W.7.

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THE experiments described in this paper were intended to test whether the hypothyroidism provoked by prolonged administration of goitrogenic substances such as thiourea affected the development of spontaneous mammary tumours in mice of high cancer strains in a way comparable to that of restricted diets. No definite conclusions were obtained with regard to the influence of the thyroid gland, but the results showed a remarkable reduction of mammary cancer in one of the two strains used. Preliminary experiments (Vazquez-Lopez, 1946; Morris, Dubnik and Dalton, 1946*a*, *b*) showed that thiourea ingestion produced a continuous anoestrus due to ovarian atrophy and the consequent underdevelopment of the mammary glands. The effect of thiourea was therefore more or less the equivalent of ovariectomy. Inasmuch as this effect depended on continuous ingestion it was decided to withdraw the drug after a certain period, thus restoring ovarian function and allowing the mammary glands to reach a stage of development comparable to that of the control animals before the average time of tumour appearance. In that case the effects observed could not be attributed to the underdeveloped condition of the gland, but could be due only to the lack of oestrogenic activity during a limited period of the life of the mice.

MATERIAL AND METHODS

C3H and R3 female mice bred in this laboratory by brother-sister mating for several years were used. The R3 mice belong to two different lines, only one of which had the high incidence of mammary cancer characteristic of this strain. The other line originated some time ago in this laboratory after mating two animals from different litters with the intention of improving breeding performance. After some generations it was found that the progeny of this pair had lost the characteristic high incidence of mammary cancer and become a low cancer line. Simultaneously with the loss of mammary cancer incidence this line developed a high incidence of lymphomas in breeding females, and thus provided the opportunity of testing the effect of thiourea in another kind of spontaneous tumour. The animals of this line (MH) used in the present experiment belonged to the 5th to 8th generations after the original cross, and most of them were born between June and September, 1946. Continuous brother-sister mating of the animals selected as descendants of the females with the earliest leukaemia development maintained the same incidence of lymphoma and mammary cancer till the 16th generation.

Breeding females only were used from the two lines of R3 mice, and both breeders and virgins from the C3H strain. The breeders were allowed to rear their first litters and, after weaning, the mothers were taken for the experiment. The litters of the C3H breeders provided the majority of the virgins employed. In all groups care was taken to divide animals of the same litters between the experimental and control groups.

The animals were housed in metal boxes, 6 to 8 in each, and fed rat cubes and water *ad lib.* Breeders and virgins of the C3H strain were separated, but the two lines of R3 breeders were mixed together.

Thiourea was administered in the drinking water, beginning with a 0·1 per cent solution and increasing the concentration as much as possible without endangering the survival of the animals. The approximate amount of fluid ingested was determined by dividing the total content of the water bottles by the number of animals in the box. All the animals were individually marked. The age of the breeders at the beginning of thiourea ingestion was between 3 and 4 months. The virgins were 3 months old, so that the mammary gland was fully developed before the action of the drug started.

The period of administration for some of the R3 animals was a year, so that most of the animals with tumours died during the period of ingestion. For the C3H mice, both breeders and virgins, it was 6 months, so that the animals were under normal conditions again when 9 or 10 months old. They were weighed regularly and inspected daily for the appearance of tumours. Vaginal smears were taken several times both during the period of thiourea ingestion and afterwards. In the virgins the presence of oestrus was also established before the start of the experiment to make sure that they had reached sexual maturity. The animals were allowed to live their full life span; only those in moribund condition were killed.

The tumours, as well as ovaries, uterus and adrenal glands of mice in the control and experimental groups were fixed for histological study. The thyroid was also examined and whole mounts of the mammary glands prepared in the usual way. The most detailed study was made in the C3H virgins of both experimental and control groups.

Effects of Thiourea.

General effects.

There were very pronounced differences between the effects of thiourea in the two strains R3 and C3H. In both lines of R3 mice, those with high and low cancer incidence, the ingestion of the 0·1 per cent solution had no effect on the appearance, weight or water intake of the animals, and the strength of the solution could be increased to 0·5 per cent and maintained for long periods. Even concentrations of 1 per cent could be used before the mice showed toxic signs such as loss of weight and reduction of water intake. If the solutions were kept between 0·2 per cent and 0·5 per cent the R3 mice could be maintained for most of their lives without any apparent difference from the controls. The average length of the experiment was 10 months.

The C3H mice, both virgins and breeders, appeared on the contrary extremely susceptible to thiourea. Some animals died during the first days of ingestion, and even with a 0·1 per cent concentration they showed marked intolerance, mainly by loss of weight, restriction of fluid intake and poor appearance. The

average weight of the animals during thiourea ingestion was 18 g. for the virgins and 21 g. for the breeders, compared with 24 and 28 g. for the control groups. The water intake was progressively reduced to a minimum of about 2 ml. per animal per day, and if the concentration of the drug was not reduced, many animals died. It was possible to reach a concentration of 0·5 per cent in most boxes, but it could be maintained only for one or two weeks. Solutions of 0·2 and 0·1 per cent were used most of the time, and even with these doses the intolerance to the drug and mounting mortality necessitated the withdrawal of the drug after 4 or 5 months. After some days without thiourea the mice recovered and the drug could then be administered again, but the recurrence of toxic symptoms was rapid. It was preferable to maintain the 6 months' period without interruption, even at the risk of losing more animals in the last months of treatment.

The doses administered were therefore variable for the different animals and for different periods for the same animal, but from the approximate estimate of water intake it may be assumed that the minimum of thiourea ingested per day was between 2 and 3 mg. for the C3H mice. For most R3 strain mice it was maintained at a level of about 15 mg. per day during most of the experiment, and during some periods it reached 30 mg. daily.

Effects on the thyroid gland.

In the R3 mice the ingestion of thiourea provoked the typical hypertrophy and hyperplasia with great vascularity, loss of colloid, etc. This effect was evident even with a 0·2 per cent solution, and became more pronounced in proportion to the length of treatment and increased concentration. As the majority of the animals with tumours died during the period of thiourea ingestion, the maximum effect was observed directly. The mice surviving after the withdrawal of the drug had thyroids of normal size and structure without any signs of the former hyperplasia.

The effects of thiourea in C3H mice were observed in the experimental animals that died during the period of ingestion of the drug. As the great majority of these mice restricted their water intake to a minimum in the weeks before their death, it could be argued that they were not actually under the action of the drug. For this reason additional mice not included in the experiment were used to test the effect under identical conditions, and were killed when the actual ingestion of thiourea was known.

In all the C3H mice of the experiment the results of the examination of the thyroid gland were similar. Thiourea administered in the drinking water did not produce hyperplasia or hypertrophy of the thyroid. There was clearly congestion of the organ which might cause a slight increase in size, but the histological picture remained normal, showing follicles filled with colloid and lined by cubic epithelium. None of the mice that died during the period of thiourea ingestion showed changes in the microscopical picture. Among 20 additional female adult mice killed after drinking solutions of 0·2 to 0·5 per cent for periods of a week to 2 months, with actual ingestion of about 5 mg. daily of thiourea, the size of the thyroid clearly increased in only one, in which also histologically the height of the cells and their disposition in solid acini without colloid corresponded to the typical picture of thyroid hyperplasia such as is found in R3 mice.

It is probable then that thiourea failed to produce any changes in the thyroid, but the possibility that some of the C3H mice reacted with hyperplasia cannot be excluded with certainty. Dalton, Morris and Dubnik (1945) and Morris *et al.* (1946*a*) found the typical hyperplasia and the expected increase in size in C3H mice fed thiourea for long periods. In a more recent paper (Dalton, Morris and Dubnik, 1948) they noted the wide individual variations in thyroid weight of thiouracil-treated mice. This lack of reaction to thiourea distinguished the C3H mice used in these experiments from the Andervont line used by Dalton *et al.* Strain differences in the thyroid reaction to thiouracil have been reported by Dickie and Wooley (1948).

Effects on oestrous cycles and mammary gland.

The effects of thiourea on the oestrous cycles differed sharply in the two strains used in these experiments. In the R3 mice both of the high and the low mammary cancer lines, the oestrous cycles remained unaffected even with the strong concentrations of thiourea which could be administered. Sometimes slight lengthening of the dioestrous intervals was observed during ingestion of 0·5 per cent and 1 per cent solutions. Most animals had normal oestrous cycles for the whole of the experiment.

C3H mice became anoestrous very soon after beginning thiourea administration and remained so during the whole time of ingestion. Smears taken at intervals during two week periods in each box were always negative. Morris *et al.* (1946*a*) found regular oestrus only in 3 mice among 21 fed thiourea. Krohn (1947), using stock albino mice injected daily with propyl-thiouracil, also found irregularities and complete disappearance of cycles. Gorbman (1947) observed an inhibitory action of thiourea on the gonads in mice. Arvy and Gabe (1946) and Pawick (1947) reported inhibition of the oestrus by thiourea and thiouracil in albino mice without any simultaneous hyperplastic action upon the thyroid after months of treatment.

Vaginal smears were also taken after discontinuing thiourea ingestion. Within a period of two weeks to a month the cycles were restored to normal. This was also the experience of Krohn (1947).

The mammary glands of the animals that died during the period of treatment showed changes correlated with the state of ovarian function. In R3 mice no difference from the controls was observed. In C3H mice absence of oestrogenic activity produced progressive atrophy with few ducts and stunted branching. Alveolar nodules were seen occasionally.

Effects on tumour incidence.

Lymphomas.—Under this title are included all the tumours of blood-forming organs, mainly lymphatic glands, thymus, spleen, etc., including the several types found in the strains with a high spontaneous incidence of such tumours.

The results of thiourea administration are given in Table I. No differences from the controls were found in the tumour incidence or in tumour age. The earlier average age of the experimental group was not significant, and the period of survival of non-tumour animals was slightly longer than in the controls. The incidence of other tumours was also the same in both groups, and that of

mammary carcinoma showed the low figures (4 per cent) characteristic of the MH line of the R3 strain.

Mammary cancer in R3 breeders.—In the original R3 line with high cancer incidence the results given in Table II are similar in the thiourea-treated mice and in the controls. The small difference in favour of the thiourea group was not significant, for the small number of animals and the lower tumour age of this group was also against any inhibitory effect of thiourea. All the tumours appeared during the period of treatment when some of the mice had received thiourea for 10 months without interruption.

Mammary cancer in C3H breeders.—Of 49 mice at the start of the experiment, only 29 (effective number) reached the age at which the earliest tumour appeared. The other 20 died as a consequence of thiourea ingestion. Eight of the 29

TABLE I.—*Lymphoma Incidence in R3 Breeding Females (MH subline).*

	Total number.	Effective number.	Mice with tumour.	%.	Average tumour age.	Average age of death in non-tumour mice.	Other tumours.
Controls	51	42	19	46	384 days (216–701)	431 days (229–730)	2 mammary cancers. 2 hepatomas.
Thiourea	55	52	22	42	321 days (146–621)	454 days (253–591)	2 mammary cancers. 1 hepatoma.

TABLE II.—*Mammary Tumour Incidence in C3H and R3 Breeding Females.*

	Total number.	Effective number.	Mice with tumour.	%.	Average tumour age.	Death age non-tumour animals.	Thyroid hyperplasia.	Period of anoestrus.
C3H controls	53	50	27	54	377 days (222–562)	421 days (242–580)	—	—
C3H thiourea-treated	49	29	8	28	457 days (367–538)	514 days (383–677)	—	+
R3 controls	7	7	7	100	435 days (301–592)	—	—	—
R3 thiourea-treated	11	10	7	70	348 days (135–442)	360 days (267–544)	+	—

developed mammary cancer. No tumour appeared during the period of treatment. The earliest developed in a mouse 367 days old, 80 days after the withdrawal of thiourea. This was only 10 days less than the average tumour age for the control group, which was 377 days. These figures confirmed the assumption that ovarian function and mammary development were normal in the experimental animals at the average tumour age of the normal mice.

In spite of these normal conditions the percentage of mammary cancer in the thiourea-treated mice was 28 per cent compared with 54 per cent for the controls. The difference is statistically significant ($\chi^2 = 3{\cdot}96$). I am indebted to my colleague, Dr. Spicer, for the statistical analysis of this and the other results of the experiment. The average tumour age was increased in the experimental animals, and so too was the average life-span of the mice which did not develop tumours compared with those in the control group. In Table II are included the relevant figures of the breeders in the high mammary cancer lines, together with some details of the effects of thiourea on thyroid hyperplasia and oestrous cycles.

Mammary cancer in C3H virgins.—Twenty-one animals died during the period of thiourea ingestion, and 64 mice survived the 6 months of treatment and reached the period of appearance of the first tumour. Five among the 64 developed mammary cancer (8 per cent), at the average age of 571 days. In the control group 40 out of 96 animals (43 per cent) developed tumours at the average age of 432 days. The earliest tumour in the controls appeared in a mouse 254 days old. The animals treated were 270 days old when thiourea was withdrawn, so that ovarian activity and mammary gland development were presumably normal not only long before the average tumour age of the controls, but also very nearly when the first tumour appeared among the normal mice. Assuming that after the end of thiourea administration the mice needed a month for total recovery, they would be comparable with the normals when 300 days old. Only 4 mammary cancers developed in the control group below this age.

The decrease in cancer incidence in the thiourea-treated virgins was highly significant ($\chi^2 = 20{\cdot}9$), and confirms the results in C3H breeders. In this group the average tumour age was also later than in the controls. The average life span of mice without tumours was longer, but with smaller differences from the control mice than in the breeders.

Microscopical Study of Organs in Thiourea-Treated C3H Mice.

A detailed study of the organs of C3H mice after longer periods of thiouracil and thiourea ingestion was published by Dalton *et al.* (1948). The animals were killed or died during treatment, and although the incidence of mammary cancer was reduced from 94 per cent to 17 per cent (Morris *et al.*, 1946*b*), the changes observed due to the continuous action of the drug may have masked those directly related to the inhibition of tumour development.

Huseby, Ball and Visscher (1945) also described alterations found in C3H mice fed restricted diets which completely inhibited mammary cancer, and Huseby and Ball (1945) those in A mice similarly treated. In both cases the restricted diets were maintained throughout the life of the animals and were effective at the time of examination. In the present experiments the withdrawal of thiourea after a limited period of administration allowed restoration of hormonal function, but the inhibition of tumours persisted. The changes observed in these circumstances should be more specifically related to the processes of cancer formation.

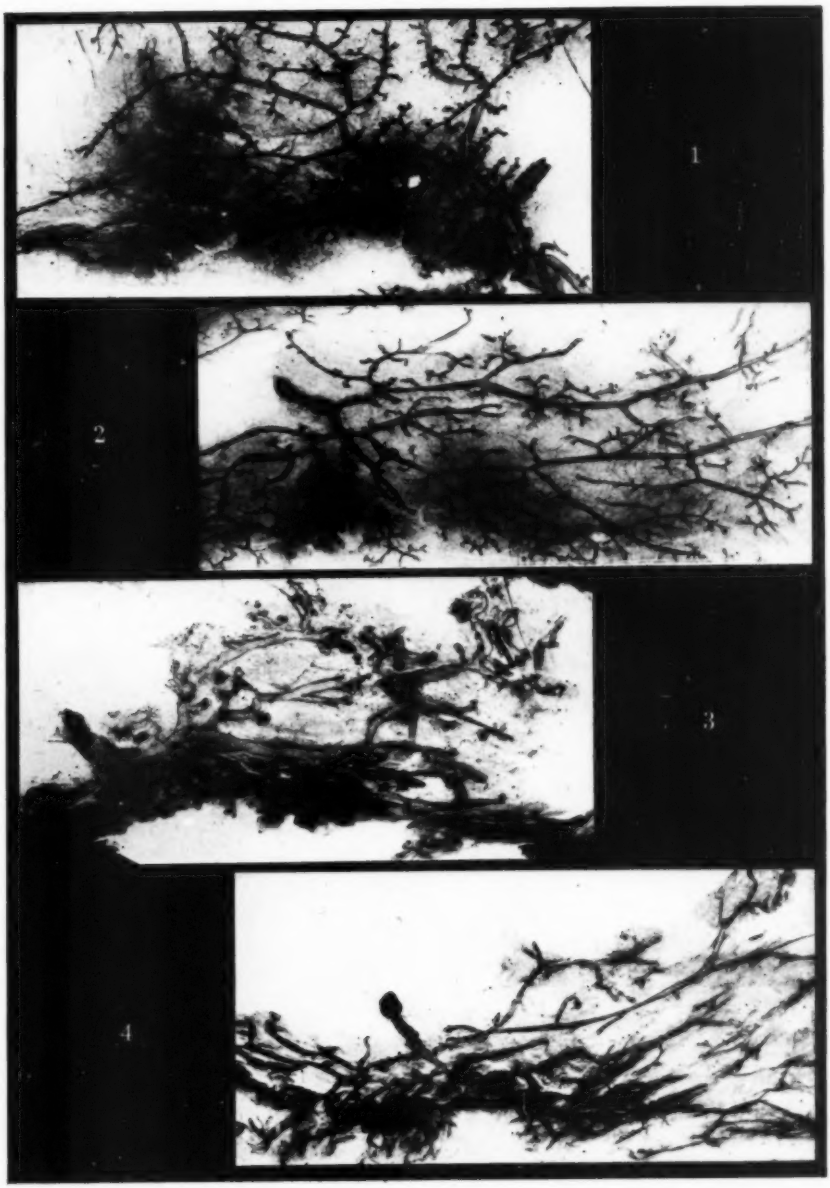
TABLE III.—*Mammary Cancer Incidence in C3H Virgins.*

	Total number.	Effective number.	Mice with mammary cancers.	%	Average tumour age.	Average death age in tumour mice.	Other tumours.
Controls	96	94	40	43	432 days (254–670)	553 days (364–716)	4 lymphoma. 1 lung cancer.*
Thiourea treated	85	64	5	8	571 days (410–673)	568 days (424–757)	4 lymphomas. 1 epithelioma.

* Animal with mammary cancer also.

EXPLANATION OF PLATE.

FIG. 1.—Mammary gland of thiourea-treated mouse. C3H virgin, 551 days old. (× 6.)
FIG. 2.—Mammary gland of control mouse of the same age. C3H virgin, 539 days old. (× 6.)
FIG. 3.—Mammary gland of thiourea-treated mouse showing great dilatation of ducts and terminal bulbs. C3H virgin, 749 days old. (× 6.)
FIG. 4.—Mammary gland of control mouse of the same age. C3H virgin, 716 days old. (× 6.)



Vazquez-Lopez.

Two groups of C3H virgins of similar ages were compared. Forty control mice between 459 and 716 days old (average 574 days) and 50 thiourea-treated, from 467 to 757 days old (average 574 days), were available for microscopical study. All were tumour-free. Tumour-bearing mice with and without thiourea treatment were compared separately, since histological changes in these mice might be secondary effects of the tumours. Ovaries, uterus, adrenal gland, thyroid and the complete set of mammary glands in each animal were studied.

Mammary glands.

In mice studied from 150 days onwards after the end of thiourea administration the mammary glands were identical to the glands of control mice of the same age in general structure, pattern, branching and extension. In both groups there was a progressive but very gradual decrease in size and richness of branching, and in most cases the development of the glands was correlated with the general condition and weight of the mouse rather than with its age. Fig. 1 and 2 show glands of control and experimental mice of approximately the same age.

The two main differences between thiourea-treated and control mice are the number of nodules of alveolar hyperplasia and the width of the ducts. The alveolar nodules of one gland or even of all the glands in a single mouse cannot be used to distinguish a thiourea-treated from a control mouse ; in some thiourea-treated animals the number and distribution of nodules is the same as in the controls. The group as a whole, however, shows significant differences, both in the number and in the distribution of nodules. The counts of the total number of nodules in tumour-free mice with and without thiourea are given in Table IV.

TABLE IV.—*Alveolar Nodules in C3H Mice (Virgin).*

	Number of mice.	Average age.	Number of mice with nodules.	%	Total of nodules.	Average number of nodules per mouse.
Controls	40	574 days (456–716)	30	75	526	17·3
Thiourea-treated	50	574 days (447–757)	39	78	310	7·3

A small proportion of animals, about the same in both groups, had none. In those mice the glands were poorly developed and almost atrophic, and although the atrophy of the mamma should not prevent the persistence of the alveolar hyperplasia, it is most probable that the absence of nodules reflects the general condition of the animals rather than any specific cause. The thiourea-treated mice, as a whole, had considerably fewer nodules of alveolar hyperplasia than the control mice, and the average number of nodules per mouse was less than half the figure for the controls.

There are no records of comparative counts in similar experiments with reduced mammary cancer incidence, but Huseby and Bittner (1946) report that less than 1 nodule per 4 glands was found in some animals more than 340 days old kept on the restricted diet in the experiments of Huseby *et al.* (1945).

The distribution of the nodules per gland given in Table V shows additional points of interest. The smaller number in the thiourea-treated mice was not due

to indiscriminate reduction affecting equally all the glands of each animal, but to a clear decrease of the number of glands with nodules. Only 32 per cent of the glands in the thiourea-treated mice had nodules compared with 51 per cent in the control group. There were also significant differences in the distribution and the average number of nodules for glands in each group, but the main effect of the thiourea administration is to increase the number of glands without any nodules. As the hormonal and systemic influences are the same for all the mammary glands in each mouse the cause of these differences between glands in the same mouse must be the result of local factors whose activity is restricted to the fields represented by particular glands. Thiourea acts systemically mainly by stopping ovarian function and oestrogen production. The effect of this lack of oestrogen, manifested in the different numbers of alveolar nodules, seems to depend on local factors independent of the general morphogenetic action.

TABLE V.—*Alevolar Nodules: Distribution per Gland.*

	Number of glands.	Number of glands with nodules.	%.	Total number of nodules.	Average number of nodules per gland.
Controls	370	190	51	526	2·7
Thiourea-treated	474	150	32	310	2

TABLE VI.—*Alveolar Nodules in C3H Mice (Virgins) with Tumours.*

	Number of mice.	Number of mice with nodules.	%.	Total number of nodules.	Average number of nodules per mouse.
Controls	18	18	100	462	25·7
Thiourea-treated	5	5	100	73	14·2

TABLE VII.—*Alveolar Nodules: Distribution per Gland.*

	Number of glands	Number of glands with nodules.	%.	Total number of nodules.	Average number of nodules per gland.
Controls	145	122	84	462	3·7
Thiourea-treated	43	36	83	73	2·0

The number of alveolar nodules in glands of control and thiourea-treated mice with tumours was also counted and the results are given in Table VI. There is a notable increase in the number compared with the tumour-free animals, but the difference between control and experimental groups persists. The distribution per gland is given in Table VII. The small number of animals makes the values of doubtful significance. The average number per gland in the thiourea-treated mice remains the same as in tumour-free animals. The percentage of glands with nodules is equal in both groups of mice without difference between thiourea-treated and controls.

The other difference between the mammary glands of the control and experimental mice affected the glandular ducts. It was not visible in all the animals, but usually, when it existed, affected all the ducts and glands of the animal to the same degree. It appeared in mice under 400 days old, and increased in frequency with age. It consisted in a considerable dilatation of the ducts, which

reached three or four times the width of the normal. It is mostly visible in the main duct, but affected the whole extension of the gland, including the end bulbs and lateral buds, which appeared almost spherical. At the post-mortem examination the ducts appeared full of whitish material, which made them conspicuous to the naked eye. It seemed therefore to be due to engorgement by a secretory product, but it could exist in glands without any indication of alveolar proliferation. Fig. 3 shows glands of treated mice with wide dilated ducts, and Fig. 4 glands of control mice of the same age.

Dilatation of ducts has been described in oestrogen-treated animals of both sexes (Burrows, 1936 ; Gardner, 1941) and by Bonser (1936) without association with proliferating acini. In cross-suckling experiments, wide dilated ducts with blunt or rounded tips, similar to those found in thiourea-treated mice, have been found in oestradiol-treated Strong A males and C57 normal females, nursed by C3H mothers (Khanolkar and Ranadive, 1947).

Other organs.

It has been already mentioned that the thyroid gland of the experimental mice did not show any alteration in histological structure in mice which previously had suffered hyperplasia during thiourea administration. The ovaries and uterus of control and treated mice did not differ in any particular. Some ovaries appeared in regressed condition without corpora lutea or follicular development ; this occurred with the same frequency in both groups. Most ovaries had follicles in all stages of development, and some of the corpora lutea became hyalinized, forming large homogeneous masses with few cells. This formation of corpora albicantis is probably a strain characteristic, since Fekete (1946) found them in the dba high cancer strain, but never in the C57 black strain. Huseby *et al.* (1945) found them only in one C3H mouse with mammary cancer, and in none of the diet restricted animals of their experiment. That is probably another of the characteristics in which the C3H mice used here differ from those in American laboratories. The corpora albicantis were very prominent among the cancer-bearing animals, and their ovaries owed their large size partly to these structures. Apart from their size, however, the ovaries of mice with tumours showed mature follicles and abundant corpora lutea, which suggested normal activity. These findings were contrary to the results of Allen, Diddle, Strong, Burford and Gardner (1935) in several strains of mice, including the C3H.

In all groups the condition of the uterus reflects that of the ovaries having tall epithelium, extensive glandular development and loose cellular stroma in the animals with well functioning ovaries and signs of regression in those with ovarian atrophy.

No differences were found in the adrenal glands of thiourea-treated mice compared with the controls. The most important change, affecting both groups equally, was the presence of amyloid degeneration, which appeared on the borderline between cortex and medulla and progressed towards the glomerular zone with advancing age. In the oldest animals amyloid invaded most of the cortex, and only two or three layers of cortical cells remained intact. The medulla was never affected. " Brown degeneration " was, on the contrary, never found in C3H mice, but was almost constant in R3 animals of high or low mammary cancer lines. Amyloid degeneration of the same type was described by Blaisdell,

Gardner and Strong (1941) in C3H mice and in other strains with and without cancer susceptibility. Dunn (1944) found it also in C3H mice and their hybrids.

The incidence and extension of adrenal amyloidosis was independent of thiourea treatment, but a difference was found between the tumour and non-tumour-bearing animals of each group, at the same age. Table VIII gives the results in non-tumour animals, 92 per cent of which showed variable degrees of amyloid invasion. Table IX shows the frequency of amyloid in animals with mammary cancer and some other spontaneous tumours. The mammary cancer group includes control and thiourea-treated mice. The percentage of tumour-bearing animals with amyloid was 41 per cent, and the difference from tumour-free mice of similar age is statistically significant ($\chi^2 = 17{\cdot}4$). It cannot be decided if this resistance to adrenal degeneration is directly related to the tumour development. The fact that tumour-bearing animals maintain better ovarian structure than non-tumour animals of the same age seems to favour instead a correlation between ovarian activity and adreno-cortical condition.

TABLE VIII.—*Amyloid Degeneration of Adrenals in C3H Virgins without Tumours.*

	Number of mice.	Age.	Number with amyloid.	%.
Controls	27	471–716 days	25	..
Thiourea-treated	25	525–749 „	23	..
Total	52	..	48	92

TABLE IX.—*Amyloid Degeneration of Adrenals in C3H Virgins with Tumours.*

	Number of mice.	Age.	Number with amyloid.	%.
Mammary cancers	16	423–715 days	8	..
Lymphoma	5	502–692 „	2	..
Epithelioma	1	590 days	..	..
Total	22	..	10	41

DISCUSSION.

From the observations described it may be concluded that the main effect of thiourea in C3H mice is the maintenance of anoestrus during the period of administration. The possibility of a temporary hypothyroidism cannot be excluded, but the significance of thyroid function for tumour production is very much reduced when it is considered that in R3 mice the incidences of mammary cancer and lymphoma are completely unaffected, in spite of considerable enlargement of the gland. If hypertrophy is accompanied by changes in the metabolic rate comparable to those taking place in rats or man, the influence of caloric intake *per se* is not very important in the genesis of malignant tumours. The possibility of a specific activity of thiourea on blood-forming tissues and their neoplasms suggested by the agranulocytic reactions in patients treated with goitrogenic substances is not supported by the results in the MH high lymphoma line which agree with clinical reports of treatment of leukaemic patients (Limarzi, Kulasavage and Pirani, 1946; Hansen-Pruss, 1947).

That lack of oestrogen is the effective factor is shown also by the normal incidence of cancer corresponding with regular oestrus in spite of thiourea administration in R3 mice. The correlation between ovarian inhibition and

decrease in mammary cancer proved by Lathrop and Loeb in 1916 is one of the oldest and probably the most often quoted experiment in hormonal cancer research. The results of ovariectomy are confirmed by the effects of hormones which antagonize oestrogenic activity such as that of testosterone (Lacassagne, 1939; Nathanson and Andervont, 1939; Lacassagne and Reynaud, 1939; Jones, 1941; Heiman, 1944). The action of progesterone is still a matter for discussion (Lacassagne, 1937; Heiman, 1945; Burrows and Hoch-Ligeti, 1946). Indirect ways of decreasing oestrogenic activity give, however, less clear results. Although low cystine diets completely inhibit mammary cancer in C3H mice (White and Andervont, 1943), the effects of stilboestrol pellets which restore the development of the mammary glands raise the incidence only to 44 per cent compared with 92 per cent in the controls (White and White, 1944). In similar experiments, Ball, Huseby and Visscher (1946) found that in spite of continuous administration of stilboestrol the mammary glands of Strain A mice maintained on restricted diets during 15 months were considerably less developed than those of control mice. The possibility of other hormonal factors influencing mammary cancer development is also suggested by the results of Shimkin and Wyman (1945), who found greater reduction in adrenalectomized C3H mice than after ovariectomy, and by the observation of Wallace, Wallace and Mills (1945) of persistence of effects of temperature in mammary cancer incidence after ovariectomy.

The results of temporary lack of oestrogenic activity caused by thiourea show that reduction of mammary cancer is not due to defective glandular development, and therefore that the hormonal effects are not limited to the morphogenesis of tissue susceptible to carcinogenic agents. Mammary glands of two groups of mice of the same strain with different cancer incidences have identical morphology, and only differ in the total number of nodules of alveolar hyperplasia in each group. The significance of these nodules of alveolar hyperplasia in relation to carcinogenesis has been indicated by several authors. The same factors involved in the genesis of mammary cancer are necessary for the alveolar hyperplasia. Huseby and Bittner (1946) have shown by the study of C3H and hybrid mice with or without the " milk influence " that this influence is necessary for the presence of nodules. The importance of the " inherited hormonal influence " is shown by the absence of nodules in virgins of the A strain. The significance of each factor for the development and persistence of the alveolar hyperplasia has been studied by Pullinger (1947), who provoked early appearance of nodules in males and ovariectomized virgins of R3 and C3H strains by injection of measured doses of oestrogens. Although alveolar hyperplasia is induced by oestrogens in mice without the milk influence, the persistence and autonomy of the alveolar hyperplasia requires the presence of the Bittner factor, and they disappear gradually in mice deprived of it. There is no nodular formation in A virgins injected with the amounts of oestrogens that are effective in other strains.

These are sufficient reasons for considering the alveolar hyperplasia as the result of the sum of hormonal and milk-transmitted influences, affecting the mammary glands of strains genetically susceptible to spontaneous tumour formation. The number of glands with nodules is considerably smaller in thiourea-treated mice. The hormonal influences in the thiourea treated mice seem to have been sufficient to reach the level necessary for the development of

nodules in the same proportion of mice as in the controls, in spite of the temporary inhibition of oestrus. The distension of ducts found also in the thiourea-treated group seems to be a sign of excessive oestrogenic stimulation, and may indicate compensatory excess after thiourea withdrawal.

If the hormonal influences are sufficient for the alveolar development the smaller number of glands with nodules, which is the main result of thiourea administration, must be due to local factors. The lack of oestrogens (and its possible compensation afterwards) affects all the glands of the mice equally. These results point to the possibility of effects on local factors and producing different consequences in each gland of the same animal. The local factor involved in the persistence of alveolar nodules is the milk factor, and the distribution of nodules suggests that it is affected by thiourea administration.

There are no grounds for supposing that thiourea can directly influence the milk factor, but there are some in favour of an indirect action resulting from the lack of oestrogens. Apart from the considerable number of experiments on the effects of oestrogen administration on cancer incidence, there is evidence even in normal circumstances of hormonal influences in breeding and forced breeding, increasing the number of tumours. The number of litters also increases the incidence in breeders (Jones, 1940; Andervont, 1941). The higher incidence in mice born in later litters compared with the earliest from the same mother has been explained by the different concentration of the milk influence with advancing age (Bittner, 1942*a*). If the increased hormonal stimulation is the cause of increase in the amount or activity of the milk influence, it is probable that the converse holds true also, and that the lack of hormones acts in the opposite sense.

The relative importance of the hormonal influence among the three factors necessary for the genesis of spontaneous mammary cancer in mice is not exactly known. Opinions range from that of Shimkin and Andervont (1941), who consider the hormonal factors as necessary only for the creation of a substratum for the carcinogenic action of the factor transmitted by the milk, to that of Warner, Reinhard and Goltz (1945), who maintain the dominance in certain cases of the inherited susceptibility upon the concentration of the milk agent. Loeb, Suntzeff, Burns and Schenken (1944) believe that the oestrogens act by stimulating the growth of the mammary tissue and sensitizing it to all types of stimuli, and Bonser (1945) as a developing factor acting upon breast tissues already sensitized by the milk factor. The only common feature of all these opinions is to consider the different factors as completely independent variables. On this basis the opinion of Bittner (1942*b*) that each influence must be considered as complementary to the others and all equally important is, besides being non-controversial, nearest to the observed facts. But if there are some factors which are affected and increased, or even created *de novo* by interaction of the others, the relative significance of each would not be the same.

The effect of genetic susceptibility on some aspects of the milk factor activities has been already proved by Heston, Deringer and Andervont (1945). The effect of the inherited hormonal influence as suggested by the result of the inhibition of oestrogenic activity would be another example of similar interaction.

SUMMARY.

1. The effects of thiourea in mice depend on strain characteristics. R3 mice, whether of high or low mammary cancer sublines, react with great thyroid

hyperplasia and without disturbance of the oestrous cycles. The incidence of lymphomas in the MH line of R3 or of mammary cancer in the original R3 strain is not affected by thiourea administration.

2. In C3H mice thiourea does not produce any thyroid reaction, but the animals remain anoestrous during the period of administration. The incidence of mammary cancer in virgins and breeding females of the C3H strain is considerably reduced by administration of thiourea during a period of six months, ending before the average tumour age of the control mice.

3. Microscopical study of the organs of tumour-free C3H mice treated with thiourea shows divergences from the control group only in the number of nodules of alveolar hyperplasia and in the width of the ducts of the mammary glands.

4. The distribution of the alveolar nodules suggests that ingestion of thiourea affects local factors involved in the development of alveolar hyperplasias.

5. Inasmuch as the effects of thiourea in C3H mice are due to the lack of oestrogenic function during the period of ingestion, the effect of hormonal factors on the genesis of mammary cancer is not limited to the development of the mammary gland, but affects also the local factors involved in the development of alveolar hyperplasia.

6. The significance of these findings is discussed, and it is concluded that the milk influence is the local factor involved in the development of mammary cancer and alveolar hyperplasia, that it is affected by the lack of oestrogens, and therefore that the "milk influence" is affected by the "inherited hormonal influence."

REFERENCES.

ALLEN, E., DIDDLE, A. W., STRONG, I. C., BURFORD, T. H., AND GARDNER, W. U.—(1935) *Amer. J. Cancer*, **25**, 291.

ANDERVONT, H. B.—(1941) *J. nat. Cancer Inst.*, **2**, 307.

ARVY, L., AND GABE, M.—(1946) *C.R. Soc. biol., Paris*, **140**, 945.

BALL, Z. B., HUSEBY, R. A., AND VISSCHER, M. B.—(1946) *Cancer Res.*, **6**, 493.

BITTNER, J. J.—(1942*a*) *Ibid.*, **2**, 540.—(1942*b*) *Ibid.*, **2**, 710.

BLAISDELL, J. S., GARDNER, W. U., AND STRONG, L.—(1941) *Ibid.*, **1**, 283.

BONSER, G. M.—(1936) *J. Path. Bact.*, **42**, 169.—(1945) *Ibid.*, **57**, 413.

BURROWS, H.—(1936) *Ibid.*, **42**, 161.

Idem AND HOCH-LIGETI, C.—(1946) *Cancer Res.*, **6**, 608.

DALTON, A. J., MORRIS, H. P., AND DUBNIK, C. S.—(1945) *J. nat. Cancer Inst.*, **5**, 451.—(1948) *Ibid.*, **9**, 201.

DICKIE, M. M., AND WOOLEY, G. W.—(1948) *Genetics*, **33**, 102.

DUNN, J. B.—(1944) *J. nat. Cancer Inst.*, **5**, 17.

FEKETE, E.—(1946) *Cancer Res.*, **6**, 263.

GARDNER, W. U.—(1941) *Ibid.*, **1**, 345.

GORBMAN, A.—(1947) *Ibid.*, **7**, 746.

HANSEN-PRUSS, O. C.—(1947) *Proc. Soc. exp. Biol., N.Y.*, **64**, 486.

HEIMAN, J.—(1944) *Cancer Res.*, **4**, 31.—(1945) *Ibid.*, **5**, 426.

HESTON, W. E., DERINGER, M. K., AND ANDERVONT, H. B.—(1945) *J. nat. Cancer Inst.*, **5**, 289.

HUSEBY, R. A., AND BALL, Z. B.—(1945) *Anat. Rec.*, **92**, 135.

Iidem AND VISSCHER, M. B.—(1945) *Cancer Res.*, **5**, 40.

Idem., AND BITTNER, J. J.—(1946) *Ibid.*, **6**, 240.

JONES, E. E.—(1940) *Amer. J. Cancer*, **39**, 94.—(1941) *Cancer Res.*, **1**, 787.

KHANOLKAR, V. R., AND RANADIVE, K. J.—(1947) *J. Path. Bact.*, **59**, 593.
KROHN, P. L.—(1947) *J. Endocrinol.*, **5**, 33.
LACASSAGNE, A.—(1937) *C.R. Soc. Biol. Paris*, **126**, 385.—(1939) *Bull. Ass. franç. Cancer*, **28**, 951.
Idem AND REYNAUD, M.—(1939) *C.R. Soc. Biol. Paris*, **132**, 431.
LATHROP, A. E. C., AND LOEB, L.—(1916) *J. Cancer Res.*, **1**, 1.
LIMARZI, I. R., KULASAVAGE, R. J., AND PIRANI, C. L.—(1946) *Blood*, **1**, 426.
LOEB, L., SUNTZEFF, V., BURNS, I. L., AND SCHENKEN, L. R.—(1944) *Arch. Path.*, **38**, 52.
MORRIS, H. P., DUBNIK, C. S., AND DALTON, A. J.—(1946*a*) *J. nat. Cancer Inst.*, **7**, 159.—(1946*b*) *Cancer Res.*, **6**, 482.
NATHANSON, I. J., AND ANDERVONT, H. B.—(1939) *Proc. Soc. exp. Biol., N.Y.*, **40**, 421.
PAWIK, W.—(1947) *C.R. Soc. Biol. Paris*, **141**, 1108.
PULLINGER, B. D.—(1947) *Brit. J. Cancer*, **1**, 177.
SHIMKIN, M. B., AND ANDERVONT, H. B.—(1941) *J. nat. Cancer Inst.*, **1**, 599.
Idem AND WYMAN, R. S.—(1945) *Ibid.*, **6**, 187.
VAZQUEZ-LOPEZ, E.—(1946) *43rd Ann. Rep. imp. Cancer Res. Fd.*, p. 10.
WALLACE, E. W., WALLACE, H., AND MILLS, C. A.—(1945) *Cancer Res.*, **5**, 47.
WARNER, S. G., REINHARD, M. C., AND GOLTZ, H. L.—(1945) *Ibid.*, **5**, 584.
WHITE, J., AND ANDERVONT, H. B.—(1943) *J. nat. Cancer Inst.*, **3**, 449.
WHITE, F. R., AND WHITE, J.—(1944) *Ibid.*, **4**, 413.

THE INFLUENCE OF THE PROTEIN CONTENT OF THE DIET ON THE RESPONSE OF WALKER RAT CARCINOMA 256 TO X-RADIATION.

L. A. ELSON AND L. F. LAMERTON.

From the Chester Beatty Research Institute, and the Physics Department, The Royal Cancer Hospital, London, S.W.3.

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IN an investigation of the body growth- and tumour growth-inhibiting action of carcinogenic substances, it was found that the nature of the inhibition is profoundly influenced by the protein content of the diet (Elson and Warren, 1947; Elson and Haddow, 1947; Elson, 1949). In rats maintained on a 20 per cent protein diet injection of the carcinogen usually has little immediate effect on body growth although a delayed action, resulting in rapid loss of weight followed by death, may occur later. On the other hand, animals maintained on a 10 per cent protein diet respond to the injection by an immediate retardation of growth.

Treatment with the carcinogenic hydrocarbon 1:2:5:6-dibenzanthracene 24 hours after implantation of Walker carcinoma 256 in rats maintained on a 20 per cent protein diet results in very little difference in size and weight of the tumours from those of controls. If, however, the animals are fed a 5 per cent protein diet the tumours of those similarly treated with dibenzanthracene were found to be only one-quarter the weight of those of controls when the rats were killed 13 days after implantation (Elson and Haddow, 1947). A much greater inhibition of tumour growth has been obtained with derivatives of 4-aminostilbene

described by Haddow, Harris, Kon and Roe (1948). Using 2′-chloro-4-dimethylaminostilbene, the tumours of the treated animals maintained on a 5 per cent protein diet reached only about 1/20th the size of those of the control animals, whereas the tumours of the treated animals fed on the 20 per cent protein diet did not differ significantly in size from controls (Elson, 1949).

From consideration of these and other results it has been suggested that the growth-inhibiting action of these substances is brought about by an interference with the availability or with the actual synthesis of proteins necessary for growth.

Since nucleic acid metabolism may be closely associated with protein synthesis (Caspersson and Santesson, 1942; Caspersson, 1947; Brachet, 1947), Elson and Harris (1947) investigated the nucleic acids in the liver of rats treated with 1:2:5:6-dibenzanthracene and found an increase in the ratio of pentosenucleic to deoxypentosenucleic acid, caused by a fall in concentration of the latter.

An initial decrease in cellular deoxypentosenucleic acid was found by Stowell (1945) after X-irradiation of transplanted mouse mammary carcinoma, and Mitchell (1942) from investigations of human tumours treated by X-radiation considered that an effect of the radiation was to inhibit the conversion of pentosenucleic to deoxypentosenucleic acid.

It appears therefore that methods by which growth inhibition is achieved by chemicals and by radiation may not be entirely unrelated. Since the inhibition of tumour growth by these chemical substances is profoundly influenced by the protein content of the diet of the animal, it becomes of considerable practical and theoretical importance to study the effect of diet on the response of tumours to treatment by X-radiation. As most of the work on tumour inhibition by these chemical substances has been carried out with the Walker rat carcinoma 256 this tumour has been selected for the present preliminary study of the effect of radiation.

EXPERIMENTAL.

Animals.—The animals used were albino rats of both sexes, weighing between 100 and 150 g. at the time of tumour implantation.

They were kept in individual cages, and maintained either on 20 per cent or on 5 per cent protein diets for at least 7 days before the tumours were implanted. The weight of each rat was recorded at intervals of 2 or 3 days throughout the period of the experiment.

Diets.—The 20 per cent protein diet was that described by Elson and Warren (1947, Table I), and the 5 per cent protein diet that described by Elson, Goulden and Warren (1947, Table II). Casein is the main protein constituent of these diets.

Radiation technique.

For the initial experiments on whole body irradiation the rats were placed in small compartments in a thin wooden box. The dosage rate at the centre of the rat's body was determined by measurements in the compartment when partly filled with Spiers' powder (Spiers, 1943). The radiation conditions were: 280 kV., filter 0·5 mm. Cu, half value layer 1·8 mm. Cu, f.s.d. 60 cm. Dosage rate at centre of rat's body, 26 r/min.

For the studies involving the localized irradiation of the tumour, it was necessary first to develop a method whereby the rat could be held firmly in reasonable comfort and the tumour alone irradiated with precision. Anaesthesia was contra-indicated, since it may affect the response of the animal to radiation.

The device eventually developed is shown in Fig. 1. A length of transparent plastic "cloth" is held by means of two clamping bars on an aluminium base. The rat is placed under the plastic material, which is then stretched under the clamping bars. The edge of the plastic cloth near the rat's head must be reinforced with a strip of cellophane one to two inches wide, which serves to hold

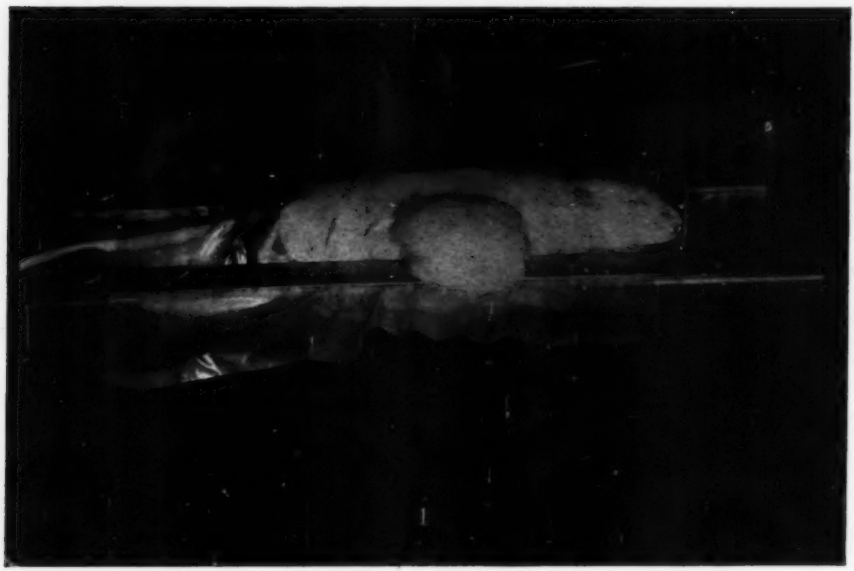

FIG. 1.—Photograph of rat in holder designed for X-ray treatment of tumour.

the rat's head down and prevents him biting his way out. The tumour itself projects through a hole in the plastic material, and can be treated by crossfire techniques without irradiation of the rest of the body.

A Victor KX10 plant was used, run at 140 kVp. with a filter of 0·1 mm. Cu, giving radiation of half value layer 0·20 mm. Cu. The applicators used were of f.s.d. 15 cm. Under these conditions the dosage rate at the centre of the tumour is about 140 r/min.

RESULTS.

Whole body X-radiation.

The experiments with whole body radiation were designed to approximate to the conditions used in tumour inhibition experiments with chemical substances (Elson and Haddow, 1947). The radiation was commenced 24 hours after tumour implantation, and thereafter daily treatments were given. Two groups

of 20 rats were used, one group being maintained on the 20 per cent protein diet and one on the 5 per cent protein diet. All the animals were implanted with Walker Carcinoma 256, and ten animals of each group kept as controls.

In the first experiment a total of 180 r per rat given in 9 daily doses of 20 r, beginning the day after tumour implantation, had no very significant effect on the growth of the tumours in animals maintained on either 20 per cent or 5 per cent protein diets. When the animals were killed 14 days after implanting the tumour the ratio (C/T) of the average weight of the tumours of the control animals (C) to that of the tumours of the treated animals (T) was 1·2 for the 20 per cent protein diet group and 0·9 for the 5 per cent protein diet group.

TABLE I.—*Effect of Whole Body X-Radiation on the Growth of Walker Carcinoma 256 in Rats Maintained on 20 per cent and on 5 per cent Protein Diets.*

Radiation conditions . . . . . 100 r given every day for eight days. Total dose = 800 r. 280 kV. Filter 0·5 mm. Cu. Half-value layer 1·8 mm. Cu.

WEIGHT OF TUMOURS AFTER 11 DAYS (*g.*).

20 per cent protein diet.		5 per cent protein diet.	
Controls (C).	Treated (T).	Controls (C).	Treated (T).
23·3	6·3	17·9	7·0
29·3	15·0	21·8	2·5
21·6	6·5	9·3	4·8
27·2	12·5	25·9	2·3
27·6	5·9	24·0	4·3
29·5	8·3	29·1	1·7
30·6	12·4	23·8	3·5
15·4	2·5	11·6	2·9
17·6	10·1	23·3	3·8
28·9	6·0	22·0	4·5
Mean *25·1*	*8·6*	*21·0*	*3·7*
$\frac{C}{T} = 2·9$		$\frac{C}{T} = 5·7$	

In a second experiment, carried out in the same manner, the total dose was increased to 800 r, given as 100 r per day for 8 days. This appeared to be close to the maximum tolerated dose. The result is given in Table I. When the animals were killed 11 days after implantation the ratio C/T was 2·9 for the 20 per cent protein diet animals and 5·7 for the 5 per cent protein diet group.

This result is similar to the results observed with chemicals in that a much greater tumour-inhibiting action is obtained in the animals maintained on the low protein diet, but the actual degree of inhibition is not as great as can be achieved by the use of certain chemicals, particularly some of the aminostilbene derivatives (Elson, 1949).

The advantage of X-ray treatment, however, is that it can be applied directly to the tumour in doses much larger than could be tolerated if applied to the whole animal, and the effect of diet, under these conditions, which approach more nearly to those used in radiotherapy of human cancer, was next investigated.

Direct irradiation of established tumours.

For these experiments the tumours were allowed to grow untreated for 6 days after implantation. Daily estimates of the size of the tumour were then made by measurement with calipers along two axes at right angles. The estimate of the area (sq. mm.) obtained by multiplying these two figures was plotted against the number of days after implantation. A typical curve for the tumour of a control animal maintained on the 5 per cent protein diet is shown in Fig. 2. From the results obtained with more than 20 control animals there appeared to be no very marked difference in rate of growth of the tumour in animals maintained either on 5 per cent or on 20 per cent protein diets. The average size of

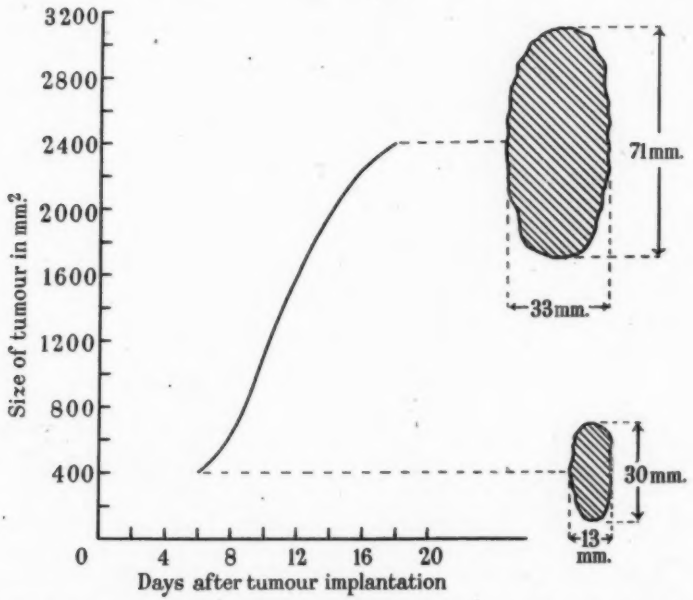

FIG. 2.—Typical growth curve of control Walker rat carcinoma 256 in animal maintained on 5 per cent protein diet, with representation of size of tumour 6 days after implantation and at death of the animal.

the tumours 6 days after implantation (when radiation was begun) was very slightly smaller in the 5 per cent protein diet animals (325 mm.²) than in the 20 per cent protein diet group (350 mm.²). In the control animals the average size of the tumours at the death of the animal was also slightly smaller (1900 mm.²) in the 5 per cent protein than in the 20 per cent protein diet animals (2300 mm.²), but the average survival time of the animals of the 5 per cent protein group was slightly shorter (17 days) than that of the 20 per cent protein group (19 days). The rate of growth of the control tumours of the 5 per cent protein group, particularly over the period during which radiation of the treated animals was carried out, was therefore very nearly equal to that of the 20 per cent protein group animals.

X-radiation, 250 *r per treatment* (*total* 4000 *r*). (Fig. 3.)

The rats received a dose of 250 r applied direct to the tumour every day except Sundays, a total of sixteen doses being given, the treatment extending over a period of eighteen days. In the group of 4 animals maintained on the 20 per cent protein diet this resulted in a slight retardation of tumour growth compared with that of controls. The average survival time was 28 days, compared with about 19 days for control animals. The group maintained on the 5 per cent protein diet, however, showed considerable inhibition of tumour growth, and the tumours after the first 7 days of treatment ceased to grow and remained fairly constant in size for a considerable time. They then, however,

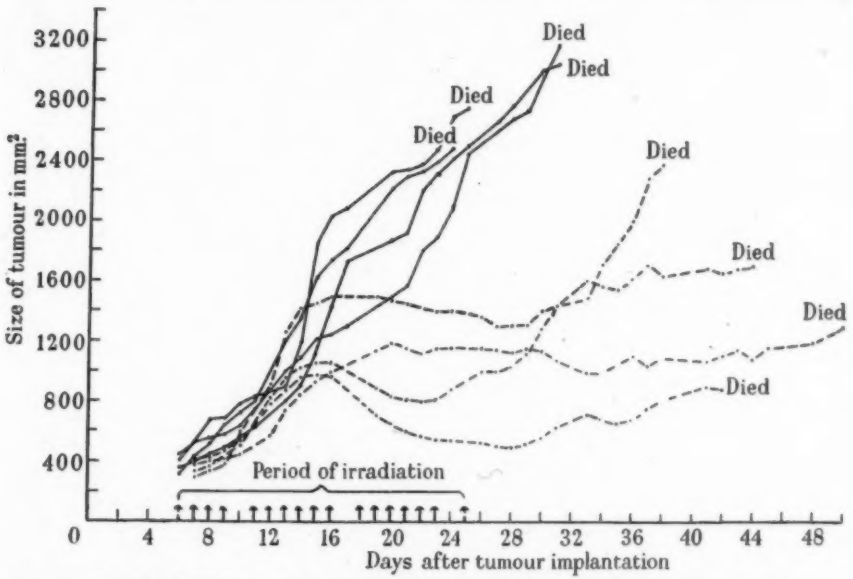

FIG. 3.—Growth of Walker rat carcinoma 256 treated with X-radiation; 250 r per day, total dose 4000 r.
—·— — · — · — Rats fed on 20 per cent protein diet.
·-·-·-·-·-·-·-·- Rats fed on 5 per cent protein diet.

gradually developed areas of necrosis, which usually become infected, and in some cases the tumour had resumed growth shortly before the death of the animal. The average survival time of the animals (44 days) was considerably longer than that of the high protein diet group. No animal in either group was cured of the tumour by this radiation treatment.

X-radiation, 400 *r per treatment* (*total* 4000 *r*). (Fig. 4.)

Since no curative effect on the Walker carcinoma was obtained with a dose of 250 r per day the rate of dosage was increased to 400 r per treatment, the total amount of radiation being still maintained at 4000 r. 400 r was given daily, except Sundays, over a total period of 12 days.

For the first four days after treatment the tumours of the 5 animals comprising the 20 per cent protein diet group grew more rapidly than those of the 5 per cent protein group, and reached an average size of about one and a half times that of the low protein group. After this period the tumours of the 20 per cent protein diet animals began to decrease in size, and eventually two of them regressed completely by a "shelling-out" process and a third remained very small over a long period. The other two, after a considerable period of regression, eventually grew again rapidly and the animals died of the tumour about 45 days after implantation.

In the 5 per cent protein group one tumour regressed completely but the others grew fairly steadily at a much reduced rate for a considerable period, eventually resuming a more rapid growth rate before the death of the animal, which occurred on the average 36 days after implantation.

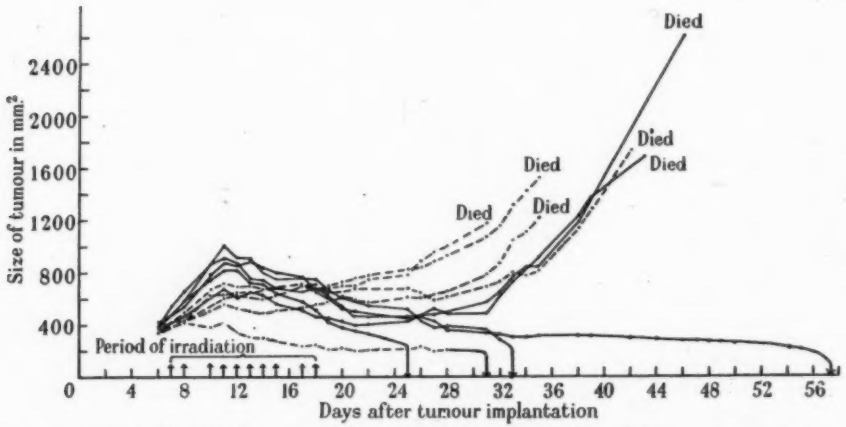

FIG. 4.—Growth of Walker rat carcinoma 256 treated with X-radiation; 400 r per day, total dose 4000 r.

———— Rats fed on 20 per cent protein diet.
-·-·-·-·- Rats fed on 5 per cent protein diet.

From this result it seems possible therefore that, although the growth-retarding action of radiation may be greater when the animals are maintained on a 5 per cent than on a 20 per cent protein diet, the degree of tumour regression and the ability of the animal to rid itself of the tumour may be favoured by a high protein diet.

This suggests that a better result might be obtained by maintaining the animals on a low protein diet during the period of radiation treatment, so achieving the greatest tumour growth-retarding effect, and then changing to the high protein diet which appears to favour regression of the inhibited tumour and cure of the animal.

This was attempted in another experiment in which the animals in the 5 per cent and 20 per cent protein diet groups were again treated daily except Sundays with 400 r. This time, however, the tumour appeared to be growing rather more rapidly than in the previous experiment, particularly in the 20 per cent

protein group. While the behaviour of the tumours of the 5 per cent protein group was very similar to that shown in the first experiment, only slight regression was observed in the 20 per cent protein group. When the regression occurred the size of all these tumours was already greater than the maximum attained by the previous 20 per cent group, and only a slight decrease in size occurred before they again resumed rapid growth. No cures were obtained in animals of either group.

It appeared therefore that regular control of the established Walker tumour could not be effected by a daily dose of 400 r, and in the next experiment the effect of a daily dose of 600 r was studied, together with the effect of changing the diet of the low protein group of animals to the high protein content at the completion of the course of radiation.

X-radiation, 600 r per treatment (total 4000 r).

In these experiments a dose of 600 r was given daily on six days, the seventh dose being reduced to 400 r to maintain the total dose at 4000 r. In the first experiment the animals of the 20 per cent protein diet group responded well at first, and nearly all the tumours began to decrease in size after the fourth dose of radiation. Although the tumours then remained small for a considerable time no complete regressions occurred, and all eventually grew again fairly rapidly, leading to the death of the animals.

The tumours of the animals maintained on the 5 per cent protein diet again at first responded to the treatment by growing rather more slowly than those of the 20 per cent protein group. The day following the last radiation treatment the 5 per cent protein group animals were changed over to the 20 per cent protein diet and maintained thereafter on this high protein diet. Under these conditions one tumour " shelled out " 30 days after implantation and one remained very small for more than 50 days, when it eventually regressed completely. The tumours of the other animals of the group remained small for a time, but then grew at about the same rate as those of the 20 per cent protein group animals.

In a repeat experiment a better response was obtained in the group of five animals maintained on the 20 per cent protein diet, and four of the tumours regressed completely. In the five animals of the 5 per cent protein diet group subsequently changed to 20 per cent protein after completion of the radiation treatment three regressions were obtained. The results of these two experiments are thus rather inconclusive.

By giving 600 r per day the total period during which the rats are under radiation treatment is, however, reduced to 8 days. Particularly in view of the poor response obtained in the first experiment with the 20 per cent protein diet group, it seems probable that this period of treatment may not be long enough to secure optimum response. However, to continue with further daily doses of 600 r, thus increasing the total beyond 4000 r, seems contra-indicated by the toxic effects which might arise since in the experiments described above two early deaths occurred not directly attributable to the growth of the tumour.

The best method of treatment of the tumours might therefore be to increase the duration of treatment by fractionation of the doses, giving larger doses early in the treatment, and to reduce the dose as the treatment proceeds and control of the growth of the tumours is being attained.

X-radiation fractionated doses (total 4000 r.). (Fig. 5 and 6.)

For the first experiment with fractionated doses (Fig. 5) the initial dose, given on the sixth day after tumour implantation, was 1000 r. In order to extend the period of treatment, and also to allow the animals to recover from this rather heavy dose, two days were allowed to elapse before the next treatment. Doses of 500 r were given on each of the following two days. No treatment was given on the next day, and then two more doses of 500 r on each of the next two days. After a further two days free from treatment one dose of 500 r was given, and followed after three days' interval by a dose of 250 r, and a final dose of 250 r after another day free from treatment. The total dosage of 4000 r was thus spread over a period of 17 days.

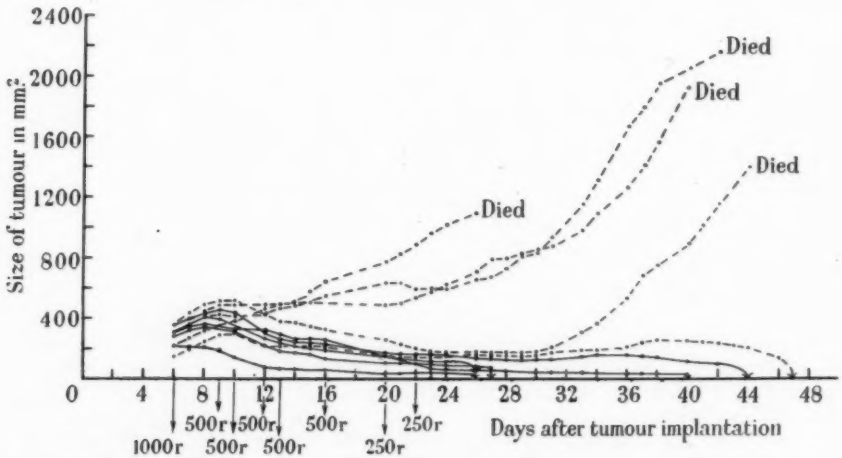

FIG. 5.—Growth of Walker rat carcinoma 256 treated with X-radiation; extended treatment with initial high dose (1000 r); total dose 4000 r.

——·——·—— Rats fed on 20 per cent protein diet.

-·-·-·-·-·- Rats fed on 5 per cent protein diet.

The growth of the tumours of all five animals maintained on the 20 per cent protein diet was rapidly brought under control and the tumours began to regress. All the animals of this group were eventually cured, complete regression occurring after about 26 days in three animals and after about 43 days in the remaining two.

On the other hand, only one of the five animals receiving similar treatment but maintained on the 5 per cent protein diet was cured. Although growth of the tumours was slowed down considerably four out of the five eventually began to grow again and subsequently caused the death of the animals. Under these conditions therefore the large initial dose of radiation appears to have such a strong inhibiting effect on the tumours that a marked difference is no longer observed between the initial response of low and high protein diet animals. The effect of the high protein diet on the process of tumour regression and on the ability of the animal to rid itself of the tumour now becomes the most important factor in deciding the response of the animal to treatment.

In a repeat of this experiment (Fig. 6) the first dose of radiation, given on the 6th day, was (by an oversight) only 300 r, and this dose was therefore followed by a dose of 1000 r on the 7th day. Doses of 500 r were then given on the 10th, 11th, 13th, 14th and 17th days, and doses of 250 r on the 21st and 24th days. The effect of the preliminary treatment with 300 r may have been to slow down the response of the tumours to the treatment. In the animals maintained on the 20 per cent protein diet regression of the tumours did not commence so early as in the previous experiment with fractionated doses, but again all the animals of this group were ultimately cured, two of the tumours regressing completely just before the 30th day and the other three before the 40th day.

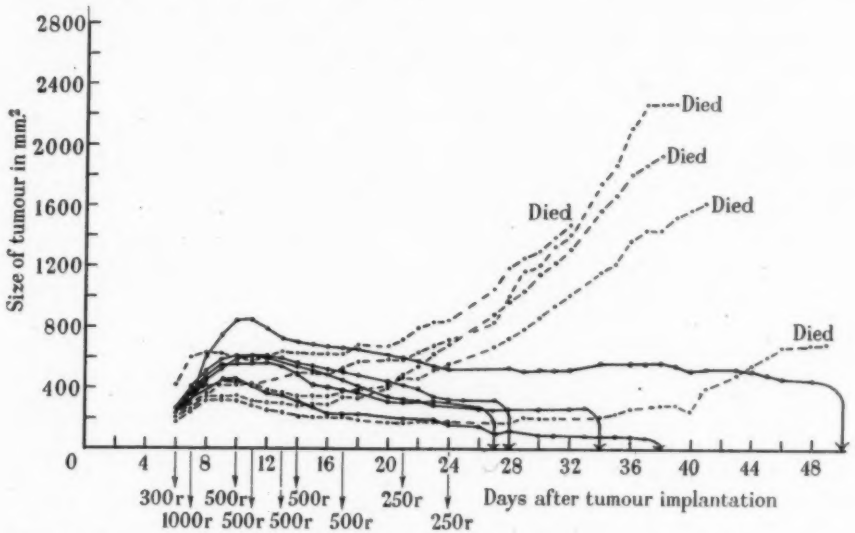

FIG. 6.—Growth of Walker rat carcinoma 256 treated with X-radiation; extended treatment with initial high dose (1000 r) following dose of 300 r, total dose 4300 r.

———— Rats fed on 20 per cent protein diet.
-·-·-·-·-·-·- Rats fed on 5 per cent protein diet.

None of the animals of the 5 per cent protein diet group in this experiment was cured of the tumour. The growth of the tumour was considerably retarded in all of them, but eventually rapid growth was resumed, which finally caused death.

In a further experiment reproducing as nearly as possible the conditions of the first fractionated dose experiment four out of the five 20 per cent protein group animals were cured, and only one cure was obtained in the five comprising the 5 per cent protein group.

The net result of the fractionated dose experiments was therefore that of 15 animals maintained on 20 per cent protein diet, 14 cures were obtained, whereas in the corresponding series of 15 animals maintained on the 5 per cent protein diet only 2 cures were effected.

DISCUSSION.

The effect of nutrition on the response of tumours to radiation appears to be a much-neglected field with regard to experimental animal studies, and indeed also in human cancer therapy. In the experiments reported in this paper, the very definite effect of the protein content of the diet on the response of the animal and the tumour to radiation treatment emphasizes the necessity of a thorough appreciation of the role of nutrition in clinical treatment.

In experiments carried out under the conditions of the "Walker tumour test," as applied to the discovery of growth-inhibitory chemicals by Haddow and his collaborators (Badger, Elson, Haddow, Hewett and Robinson, 1942; Haddow, Harris, Kon and Roe, 1948), Elson and Haddow (1947) and Elson (1948) found that the inhibiting action of these chemicals could be largely neutralized by maintaining the animals on a high protein diet. The greatest degree of tumour inhibition was obtained when the animals were maintained on a low protein diet.

It has now been shown that the response of rats implanted with Walker carcinoma 256 to whole body X-radiation under conditions approaching those of the "Walker tumour test" is also greater in those animals maintained on the low protein diet. The protective action of the high protein diet on the inhibition of tumour growth by whole body radiation is, however, not so great as is observed in treatment with some of the chemical inhibitors.

The "Walker tumour test" can be considered as a very efficient sorting test designed to reveal the maximum tumour growth-inhibiting action of the particular substance under test, the compound being usually given by intraperitoneal injection within 24 hours of the implantation of the tumour. Thus the conditions under which this test is carried out are necessarily far removed from those existing in the therapy of human cancer.

It was therefore decided to approach more closely to clinical conditions by allowing the tumour to become well established, and to grow to a definite size before treatment was commenced, the response of the tumour being followed by daily measurements of tumour size. This technique has been adopted in the present studies of the localized irradiation of tumours, and it is hoped that its use will also eventually allow a direct comparison to be made between the action of radiation and that of chemicals suitably selected by the sorting test, and make possible a study of the combined effect of chemotherapy and radiotherapy.

The experiments on the localized irradiation of established tumours reveal a greater inhibitory action in the animals maintained on the low protein diet than in those maintained on a high protein diet, the difference in response being most marked when the radiation is applied in frequent small doses (250 r daily, total dose 4000 r, Fig. 3), although this form of treatment rarely leads to complete regression of the tumour. However, if the same total dose is applied in the form of larger daily doses, particularly if an initial dose of 1000 r is given, with extended time intervals between successive smaller doses (Fig. 5 and 6), regression of many of the tumours can be achieved. The number of regressions and cures is then found to be much greater in the animals maintained on the high protein diet.

It is clear therefore that in considering the response to radiation of the tumour, one must separate to some extent the processes which determine—

(*a*) The initial response of the tumour.

(*b*) The elimination of the inhibited tumour and cure of the animal.

Process (*a*) is found to be favoured by a low protein diet, and process (*b*) by a high protein diet.

In work on the inhibition of tumours by chemicals, the first process, (*a*), has, in general, been the one observed, and it is now seen that the effect of diet on this process is similar for both X-radiation and chemical treatment. With X-ray treatment, however, it is possible to apply direct to the tumour a sufficiently large dose of radiation to bring about an adequate initial growth-inhibitory response, even in animals maintained on a high protein diet, so that the subsequent outcome of the treatment then depends almost entirely on process (*b*), and a favourable result is thus to a very large extent dependent on a high protein content of the diet.

Though the results obtained may be related to some extent to the particular characteristics of the tumour used, it is very interesting to note how closely the two processes distinguished in the radiation response of the tumour follow the concepts of "intra-cellular response" and "inter-cellular response" put forward by Koller and Smithers (1946) from studies in clinical radiotherapy. Koller and Smithers suggest that ". . . Tumour regression as the result of irradiation is initiated by an intra-cellular response, but is carried on by an inter-cellular response which follows and plays an important part in the success of any treatment. The intra-cellular reaction is directly responsible for the death of individual cells, and its rate is of particular importance at the beginning of treatment. The inter-cellular reaction is manifested through the repair processes, and depends on the construction of the tumour tissue as a whole. This reaction is conditioned and influenced by the intra-cellular response; in this sense it is an indirect effect of radiation . . ."

It seems likely that this "inter-cellular" response may constitute an important part of what we have described as process (*b*), and have shown to be favourably influenced by a high protein diet. If the "inter-cellular response" is related to the repair processes, then it is very reasonable to suppose that nutritional factors can affect the elimination of the tumour in radiation treatment, and indeed proteins would be of special importance in view of their known value in the healing of burns and wounds.

Clearly the present work can only be regarded as a preliminary study. A number of different lines of investigation have now to be followed, including the question of whether a certain type of protein or any of its constituent amino-acids are particularly concerned, and also whether other nutritional factors can contribute to the effectiveness of the treatment. Similar investigations with other animal tumours must also be carried out. For the fuller appreciation of these results a systematic histological and cytological study of the tumour at various stages of the radiation treatment is essential, and this study is now under way.

SUMMARY.

The effect of diets containing 20 per cent and 5 per cent protein respectively on the response of Walker rat carcinoma 256 to whole body X-radiation and to localized irradiation of the tumour has been investigated.

For whole body radiation (100 r daily for 8 days commencing 24 hours after tumour implantation) or for localized irradiation of the established tumour with 4000 r total dose applied in daily doses of 250 r, commencing 6 days after tumour

implantation, inhibition of tumour growth was greatest in those animals maintained on the low protein diet, but no complete regressions of the tumour were obtained.

With localized irradiation of the tumour, when the total dose of 4000 r was applied in larger daily doses, complete regression of some tumours was observed, and the most favourable results were obtained by application of an initial dose of 1000 r with extended time intervals between successive smaller doses. Complete regression of the tumours was then obtained in about 90 per cent of the animals maintained on the high protein diet, but in only about 15 per cent of these maintained on the low protein diet.

It is suggested that two effects are concerned in the response of tumours to radiation, (*a*) the initial inhibition of tumour growth, (*b*) the elimination of the inhibited tumour and cure of the animal. Process (*a*) is favoured by a low protein diet, and process (*b*) by a high protein diet.

We would like to express our appreciation of the interest taken in this work and the encouragement given by Professor A. Haddow and Professor W. V. Mayneord, and of the able assistance of Miss C. B. Barrett, Miss M. Winsborough and Mr. R. Poppy. We also wish to express our thanks for grants supporting this investigation from the British Empire Cancer Campaign, the Anna Fuller Fund, the Jane Coffin Childs Memorial Fund for Medical Research and the U.S. Public Health Service; also for facilities provided by Imperial Chemical Industries Limited.

REFERENCES.

BADGER, G. M., ELSON, L. A., HADDOW, A., HEWITT, C. L., AND ROBINSON, A. M.—(1942) *Proc. Roy. Soc.*, B, **130**, 255.

BRACHET, J.—(1947) *Cold Spring Harbor Symposia on Quantitative Biology*, **12**, 18.

CASPERSSON, T.—(1947) *Symp. Soc. exp. Biol.*, **1**, 127.

Idem AND SANTESSON, L.—(1942) *Acta radiol. Stockh. Suppl.*, 46.

ELSON, L. A.—(1948) *Acta Union int. Cancer*, **4**, 396.—(1949) *Symp. Soc. exp. Biol.*, **3**, 327.

Idem AND HADDOW, A.—(1947) *Brit. J. Cancer*, **1**, 97.

Idem AND HARRIS, R. J. C.—(1947) *Ibid.*, **1**, 327.

Idem AND WARREN, F. L.—(1947) *Ibid.*, **1**, 86.

Idem, GOULDEN, F., AND WARREN, F. L.—(1947) *Ibid.*, **1**, 80.

HADDOW, A., HARRIS, R. J. C., KON, G. A. R., AND ROE, E. M. F.—(1948) *Phil. Trans.*, A, **241**, 147.

KOLLER, P. C., AND SMITHERS, D. W.—(1946) *Brit. J. Radiol.*, **29**, 89.

MITCHELL, J. S.—(1942) *Brit. J. exp. Path.*, **23**, 296, 309.

SPIERS, F. W.—(1943) *Brit. J. Radiol.*, **16**, 90.

STOWELL, R. E.—(1945) *Cancer Res.*, **5**, 169.

THE EFFECTS OF INJECTION OF PURINE AND PYRIMIDINE COMPOUNDS ON GRAFTED MICE.

G. R. BARKER, M. M. DHAR AND L. D. PARSONS.

From the Department of Chemistry, The University, Manchester, 13, and the Royal Eye Hospital, Southwark, London.

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THE effects of injection of the four nucleotides obtained from ribonucleic acid on the tissues of normal and tumour-bearing mice have been described (Barakan, Barker, Gulland and Parsons, 1948; Parsons, Gulland and Barker, 1947). Further experiments have now been carried out to note the action of purine and pyrimidine bases on sarcoma growth, and to correlate if possible the biological activities of the compounds with their chemical structure. These experiments fall into two groups:

(*a*) Those relating to the amino and hydroxy purines,
(*b*) those relating to uracil and its derivatives.

Both pure-line and stock mice were grafted with homologous or heterologous sarcomas, including C57 and S37 and the Crocker sarcoma, the latter known to be very refractory to inhibitory action (Badger, Elson, Haddow, Hewett and Robinson, 1942). The technique for treatment of the grafted mice was similar to that previously described, but the standard dose (0·025 g.) was reduced in proportion to the toxicity of any particular compound, or where the condition of the mouse necessitated this. Insoluble or relatively insoluble compounds such as xanthine, guanine, adenine or hypoxanthine were administered as fine suspensions in the same media as used for the soluble compounds. Examination of the tissues at varying intervals showed that all compounds injected as suspensions were rapidly removed within 24 hours with the exception of guanine, the relative inactivity of which on tumour growth seems to be due to its marked insolubility. Deposits of the injected guanine were found at the site of injection long after the last dose. Table I summarizes the results obtained. The total number of control mice was in all cases the same as the total number of treated mice.

The amino and hydroxy purines.

It had been noted that adenylic acid or adenosine produced shock when injected into mice, and it seems probable that this effect was due to the adenine radical, of which the pyrimidine ring is in a lower state of oxidation than those of the other compounds used which do not induce shock. Injection of adenine in doses comparable with those used for adenosine or adenylic acid has been found to cause immediate and profound shock with heavy mortality, the minimum sublethal dose being 0·005 g. On the other hand, hypoxanthine injected in average doses of 0·015 g. produced no shock. It is seen from Table I that this purine markedly inhibits sarcoma growth. Toxic symptoms appear on repeated dosage with considerable mortality.

These experiments suggest that the property of inducing shock shown by adenylic acid is dependent on neither the phosphoryl nor the carbohydrate

radical, but may be connected with the presence of the amino group in position 6 in the adenine radical.

Xanthine injected in tumour-bearing mice was removed from the tissues in 24 hours, but no inhibition of sarcoma growth was observed. Injected into normal mice in doses of from 0·02–0·025 g. 5 times weekly, xanthine induces, after the first or second treatment, a moderate eosinophilia which is also characteristic of guanylic acid. It is interesting to note that uridylic acid, which, like xanthine, contains the dihydroxypyrimidine ring system, also induces a moderate eosinophilia in normal mice. It would therefore seem that the amino group in position 2 of guanine is not essential for the production of eosinophilia.

TABLE I.

Compound used.	No. of mice treated.	T. Total weight of treated tumours (g.).	C. Total weight of control tumours (g.).	Ratio T./C.
Adenine sulphate	7	11·9	19·4	0·614
Adenine	10	13·1	24·2	0·542
Adenosine	6	9·7	14·7	0·66
Guanine hydrochloride	14	26·6	35·9	0·741
Guanine	6	13·9	15·5	0·897
Guanosine	5	4·5	10·0	0·45
Hypoxanthine	18	17·1	35·1	0·488
Xanthine	15	73·2	75·0	0·964
Uracil	18	80·4	66·3	1·21
4-aminouracil	61	65·8	139·8	0·471
4-methyl-uracil	40	96·4	85·7	1·13
Orotic acid	17	38·4	39·4	0·975
4:5-diamino-uracil	8	21·1	25·7	0·82
4-amino-5-methyl-uracil	30	76·1	66·2	1·15
4-amino-uracil with guanylic acid	11	19·0	28·1	0·676

Uracil and related compounds.

Previous work (Barakan *et al.*, 1948) has shown that uracil, uridine or uridylic acid behave differently to the purine compounds in their effect on tumour growth, and exert a stimulatory action on the development of mouse sarcoma. This difference in behaviour may be due to the different ways in which the injected compounds are metabolized. It is known (Brown, Petermann and Furst, 1948; Getler, Roll, Tinker and Brown, 1949; Arvidson, Eliasson, Hammarsten, Reichard, von Ubisch and Bergstrom, 1949) that, whereas ingested adenine is utilized in the synthesis of pentose nucleic acid, uracil is not, and that orotic acid is probably a precursor of uridine and cytidine. Furthermore, neither xanthine nor hypoxanthine are utilized in polynucleotide synthesis. It therefore seemed desirable to investigate further the biological effects consequent on substituting various groups in the uracil molecule, and a number of compounds were accordingly synthetized, including orotic acid and other derivatives, which might act as purine precursors.

As seen from Table I, orotic acid has no observable action on tumour growth.

4:5-diamino-uracil administered in the form of a salt, or as the freshly-prepared base, exhibited high toxicity, and treatment was discontinued after a few doses. 4-aminouracil, when injected in aqueous solution in stock and pure line mice, produced considerable inhibition of the grafted tumour, statistical analysis showing that the differences in tumour weights for treated and control mice were significant. Of the tumours employed, the Crocker showed the greatest inhibition, whereas this sarcoma was very slightly affected by adenylic or guanylic acid.

The possibility that the inhibitory action of 4-aminouracil may be due to the utilization of this compound in some synthetic process in cell metabolism leading to the formation of a purine demands further investigation. In this connection it is significant that 4-methyl uracil and 4-amino-5-methyl uracil, in which purine synthesis is blocked by the methyl group, have been found to have no inhibitory action on sarcoma growth, and the marked difference in the actions of 4-amino-uracil and uracil itself on tumour development is worthy of emphasis. This difference in behaviour of these compounds is further shown by the fact that inhibition of tumour growth was noted when guanylic acid and 4-aminouracil were injected together in grafted mice, whereas it had been previously observed that guanylic and uridylic acids were mutually antagonistic, and that their respective inhibitory and acceleratory actions were cancelled out when injected simultaneously.

Experiments are in progress to examine other aspects of the relationship of purine and pyrimidine compounds to sarcoma growth.

SUMMARY.

Previous observations regarding the effects of nucleotides and related compounds on the tissues of normal and tumour-bearing mice have been extended. Adenine is found to be highly toxic, but hypoxanthine, whilst inhibiting sarcoma growth, is toxic only on continued dosage. Xanthine has no effect on sarcoma growth, but, like guanylic acid, produces eosinophilia. Of the three pyrimidine bases investigated, orotic acid showed no action on tumour growth; 4:5-diaminouracil was too toxic to allow of continued dosage; 4-aminouracil showed a significant inhibiting effect on grafted tumours, the greatest effect being observed with the Crocker sarcoma.

We are grateful to the British Empire Cancer Campaign for grants defraying the cost of these investigations.

REFERENCES.

ARVIDSON, H., ELIASSON, N. A., HAMMARSTEN, E., REICHARD, P., VON UBISCH, H., AND BERGSTROM, S.—(1949) *J. biol. Chem.*, **179**, 169.

BADGER, G. M., ELSON, L. A., HADDOW, A., HEWETT, C. L., AND ROBINSON, A. M.—(1942) *Proc. Roy. Soc.*, B, **130**, 255.

BARAKAN, T. H., BARKER, G. R., GULLAND, J. M., AND PARSONS, L. D.—(1948) *J. Path. Bact.*, **60**, 441.

BROWN, G. B., PETERMANN, MARY L., AND FURST, S. S.—(1948) *J. biol. Chem.*, **174**, 1043.

GETLER, HELEN, ROLL, P. M., TINKER, J. F., AND BROWN, G. B.—(1949) *Ibid.*, **178**, 259.

PARSONS, L. D., GULLAND, J. M., AND BARKER, G. R.—(1947) *Symp. Soc. exp. Biol.*, **1**, 179.

FURTHER EXPERIMENTS ON THE *IN VITRO* METABOLISM OF 3 : 4-BENZPYRENE IN THE MOUSE SKIN.

A. K. POWELL AND G. CALCUTT.

From the Department of Cancer Research, Mount Vernon Hospital, and the Radium Institute, Northwood, Middlesex.

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A TECHNIQUE whereby the metabolism of 3 : 4-benzpyrene in mouse skin could be followed under *in vitro* conditions was described by Weigert, Calcutt and Powell (1946). The original experiments were carried out with Ringer Locke solution as the nutrient medium. Under these conditions measurable quantities of the metabolite BpX_2 were found, but in all cases the amounts concerned were very low. Since further alternative techniques failed to offer sufficient of the metabolite for chemical analysis (Powell and Calcutt, 1947), an attempt was made to improve the yield from the *in vitro* system by varying the nutrient medium. The results were a failure in this respect, and at the time no satisfactory explanation of them was available. However, in the light of more recent work—Calcutt (1949)—it is now possible to proffer a reasonable hypothesis.

MATERIALS AND METHODS.

The skins used in these experiments were all taken from comparable positions on the flanks of 12-week-old male pure line Strong A mice. The technique for painting with the 0·3 per cent benzpyrene solution in acetone was that described by Calcutt and Powell (1947), and further treatment was as in earlier work (Weigert, Calcutt and Powell, 1946). Extraction and estimations of the metabolites were by the methods of Weigert (1948).

The nutrient media used were as below :

1. Ringer Locke Solution.
2. Tyrode Solution.
3. Hédon-Fleig Saline.
4. Pannett-Compton Saline.
5. Sterilized tap water.
6. Normal Saline.

All media were made up to formulae given in the 'Microtomists' Vade Mecum' (Lee, 1937).

In all experiments incubation was at 37° C. for a period of 8 hours.

RESULTS.

Metabolism of the benzpyrene only occurred in the series on the four buffered media. In the case of the tap water and the normal saline the media were found to be turbid and the skins sodden and waterlogged. No indications of any

change in the benzpyrene could be found in these cases, and it appeared that the conditions were too unfavourable for the survival of the skins. These have, therefore, been left out of further consideration.

With the buffered media metabolism took place and measurable quantities of BpX_2 were extracted. The results are shown diagrammatically in Fig. 1, the amounts of metabolites being estimated as μmg. of BpX_2. It is apparent that the metabolic turnover has shown a tendency to decrease as the pH of the medium has increased.

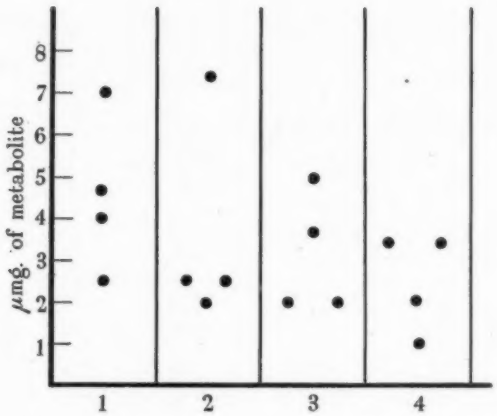

FIG. 1.—Metabolism of 0·3 mg. of 3:4-benzpyrene on 4 sq. cm. of mouse skin on various media.
(1) Ringer-Locke Solution –pH 7·3.
(2) Pannett-Compton Saline –pH 7·5.
(3) Tyrose Solution –pH 7·8.
(4) Hédon-Fleig Saline –pH 8·8.

DISCUSSION.

As an attempt to produce benzpyrene metabolites in greater amount these experiments must be considered a failure. On the other hand, the variation in the yields obtained is in itself of some interest. Explanations of this based on the detailed composition of the media used appear to be of doubtful significance. The outstanding point seems to be the pH of the medium.

Under the conditions of experiment it is reasonable to assume that the tissues of the skins concerned became adjusted in some degree to the environmental conditions, that is, that the internal state of the cells approximated more nearly to the external pH than would normally be the case. The question arises, therefore, as to whether such a state of affairs could influence the metabolic turnover.

Taking advantage of some recent findings, it is believed that an explanation of the results can be offered along the above lines.

It has long been suspected that the process of carcinogenesis is intimately involved with sulphydryl groups. This view has recently been reinforced by Crabtree (1948), and the weight of evidence is now strongly in its favour. At the same time it has been suggested that the metabolism of benzpyrene is also mediated by –SH groups. This has received confirmation by the findings of

Calcutt (1949) that benzpyrene will react directly with –SH-containing compounds, and that, furthermore, the reaction products of such systems include compounds which in their physical and spectrographic properties show a remarkable resemblance to the metabolites from animals. If, then, this be accepted, the variation in the results found above would imply some variation in either the availability of –SH for metabolic purposes, or some other factor influencing the rate of reaction.

It is well recognized that variations in pH will effect the degree of denaturation of proteins—a movement of pH towards the acid side favouring denaturation. The consequences are a splitting of certain molecules and the appearance of free –SH groups (Mirsky, 1941).

On the basis of the previously suggested movement of the internal pH of the tissues concerned in order to approximate to external conditions it would be expected that the availability of –SH groups would also vary, being greater in the more acid conditions.

It follows, therefore, that the decline in metabolic turnover as the conditions move to the alkaline side is completely in keeping with the view that metabolism of 3:4-benzpyrene is dependent upon sulphydryl.

SUMMARY.

1. The metabolism of 3:4-benzpyrene in mouse skins has been followed under *in vitro* conditions using several culture media.

2. It is found that metabolic turnover decreases as the pH of the medium increases.

3. It is reasoned that these findings are in accord with the hypothesis that the metabolism of benzpyrene is mediated by sulphydryl groups.

REFERENCES.

CALCUTT, G.—(1949) *Brit. J. Cancer*, **3**, 306.
Idem AND POWELL, A. K.—(1947) *Ibid.*, **1**, 323.
CRABTREE, H. G.—(1948) *Ibid.*, **2**, 281.
LEE, A. B.—(1937) 'Microtomists' Vade Mecum,' 10th ed. London (Churchill).
MIRSKY, A. E.—(1941) *J. gen. Physiol.*, **24**, 709.
POWELL, A. K., AND CALCUTT, G.—(1947) '24th Ann. Rep. Brit. Emp. Cancer Cmpgn.,' p. 87.
WEIGERT, F.—(1948) *Cancer Res.*, **8**, 169.
Idem, CALCUTT, G., AND POWELL, A. K.—(1946) *Nature*, **158**, 467.

SURVEY OF PAPERS.

HEADY AND KENNAWAY examine some of the evidence offered on the question whether the recent increase in deaths attributed to cancer of the lung is due to a real increase in the incidence of this disease. They suggest that, in assessing the incidence upon the whole population from hospital statistics, the best index is the ratio of autopsies from cancer of the lung to total autopsies (p. 311).

UNGAR describes carcinoma of the duodenum with concomitant polyposis occurring in two brothers and a sister, 16, 18 and 19 years of age, respectively. The implications of this observation for the understanding of malignant transformation in intestinal polyposis are discussed (p. 321).

GUNZ describes the effects of X-radiation on cultures of leucocytes from patients with chronic myeloid leukaemia. Mitosis was temporarily inhibited by a dose of 100 r and apparently permanently by 1000 r. There was no evidence that X-rays acted by direct destruction of resting cells (p. 330).

FOULDS has studied the growth of 275 spontaneous mammary tumours in hybrid mice from their first appearance till the death of the mice. Independently of their intrinsic growth rate the tumours could be divided into two main classes: responsive tumours which grew during pregnancy and thereafter regressed and unresponsive tumours which grew steadily. Sudden changes in behaviour, often abrupt, were also observed in some tumours (p. 345).

DMOCHOWSKY AND ORR have obtained breast tumours in C3H high-breast-cancer strain male mice and in one C57 Black low-breast-cancer strain male mouse following application on the skin of methylcholanthrene and oestrone. Methylcholanthrene alone had no such effect, whereas oestrone alone gave breast tumours in C3H males but not in C57 Black males (p. 376).

BERENBLUM AND SHUBIK, continuing their work on the development of skin tumours in mice from latent tumour cells induced by 9 : 10-dimethyl-1 : 2-benzanthracene, now find that croton oil, when applied 43 weeks after a single painting of the skin with the carcinogen, elicits a tumour incidence similar to that occurring when the interval is 3 weeks or less (p. 384).

CRABTREE has tested six isomeric aminoazotoluenes for their carcinogenic activity on the livers of mice and rats by oral administration. Two are carcinogenic in the rat and do not influence the growth-rate of the rats, while the others, which have *p*-toluidine as a split-product, are non-carcinogenic, but stimulate growth. A possible relationship between folic acid metabolism and the mechanism of cancer induction is suggested (p. 387).

BRUZZONE finds that the dosage of progesterone necessary to prevent the formation of abdominal fibroids in guinea-pigs after the administration of diethylstilboestrol and hexoestrol is similar to that required when α-oestradiol is used as the inducing agent (p. 398).

VAZQUEZ-LOPEZ finds that the oral administration of thiourea to R3 mice causes great thyroid hyperplasia without disturbance of oestrous cycles and has no effect on the incidence of spontaneous tumours. In C3H mice there is no thyroid reaction, but there is persistent anoestrus and a considerable reduction in tumour incidence. The significance of these findings in relation to the milk factor is discussed (p. 401).

ELSON AND LAMERTON have studied the effects of high and low protein diet on the response of a transplantation tumour in the rat to whole body X-radiation and localized irradiation of the tumour. Their findings suggest that the initial inhibition of tumour growth is favoured by a low protein diet and the elimination of the inhibited tumour is favoured by a high protein diet (p. 414).

BARKER, DHAR AND PARSONS have investigated the effects of purine and pyrimidine bases on the growth of transplanted tumours in mice. 4-Aminouracil showed a significant inhibiting effect (p. 427).

POWELL AND CALCUTT have investigated the metabolism of benzpyrene in mouse skins under *in vitro* conditions. They find that the metabolic turnover decreases as the pH of the medium increases, and consider that their results are in accord with the hypothesis that the metabolism of benzpyrene is mediated by sulphydryl groups (p. 430).